Mechanisms of Hard Tissue Destruction

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Mechanisms of Hard Tissue Destruction

A Symposium Presented at the Philadelphia Meeting of The American Association for the Advancement of Science December 29 and 30, 1962

Edited by

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Preface

Mechanisms of Hard Tissue Destruction is based on a four-session symposium organized by the Section on Dentistry of the American Association for the Advancement of Science, held during the 129th annual meeting of the AAAS in Philadelphia, Pennsylvania, on December 29 and 30, 1962.

The symposium was cosponsored by the AAAS Sections on Dentistry (Nd), Medicine (N), and Zoology (F) and by the International Association for Dental Research, North American Division, the American College of Dentists, and the American Dental Association.

A multidisciplinary approach was chosen with a view to covering a theme which could serve as a logical sequence—the other side of the coin, as it were—to a previous AAAS symposium which dealt with the formative aspects of hard tissue biology (*Calcification in Biological Systems,* AAAS Publication No. 64, Washington, D. C., 1960).

In the present volume the oral presentations have been supplemented by three additional manuscripts (chapters 5, 10, and 23), making a total of twenty-six chapters by forty-eight authors and coauthors, including fourteen from institutions outside the United States.

The international participation in this symposium was made possible in part by a conference grant from the National Institute of Dental Research, negotiated together with Dean Lester W. Burket and Dr. Ned B. Williams, University of Pennsylvania School of Dentistry, the latter serving as 1962 AAAS Vice President and Chairman of AAAS Section on Dentistry (Nd).

Serving with me as co-chairmen of the individual symposium sessions were Drs. Seymour J. Kreshover, National Institute of Dental

Research (now Secretary, AAAS Section Nd); Franklin C. McLean, University of Chicago; and George Nichols, Jr., Harvard University.

The cost of the color plate (facing page 218) was covered by a grant from the Miami Valley Laboratories of the Procter & Gamble Company, Cincinnati, Ohio.

From the initial planning until the final preparation of subject and author indexes, I have enjoyed assistance beyond the hours of duty from Mrs. Dorothy Good, Administrative Assistant, and Mrs. Phyllis Lessin, Secretary, School of Dentistry of the University of California, Los Angeles.

I am grateful for the excellent cooperation of all the authors here and abroad, and for other expert help in editing this volume.

Each of the individual chapters that follow is concluded with a summary, and the brief tabulation below may serve to orient the

SUMMARY OF CONTENTS

Chapter numbers	Destructive processes	Structures involved	Biological influences
1 to 4	Boring canals	Calcareous rocks Coral reefs Shells Tusks and teeth	Mollusks Sponges Gastropods Postmortem fungi (?)
4 and 5	Attrition	Teeth	Mastication, bruxism
	Abrasion	Teeth	
	Erosion	Enamel, dentin	Saliva, bacteria (?)
	"Erosion"	Enamel	Postmortem algae (?)
6 to 10	Caries	Teeth	Bacteria
10 to 24	Resorption	Antler Bone Cementum Dentin Enamel	Multinucleated giant cells
	"Osteolysis"	Bone	Osteocytes
25	Chelation	Shells, bones, teeth (?)	Sequestering agents
26	Proteolysis	Collagen	Collagenase

reader regarding the extent to which different reports have shed light on related mechanisms.

The primary purpose of the symposium was to examine the conditions in which mineralized structures—including rocks, corals, shells, antlers, bone, ivory, cementum, dentin, and enamel—are subject to destruction by various marine and subterranean organisms such as boring sponges, mollusks, snails, octopuses, worms, algae, and fungi, as well as by the action of the giant cells typical of lacunar resorption and the oral bacteria responsible for tooth decay. Beyond the morphological and cellular levels of observation, the symposium also served to delineate present knowledge and various areas of ignorance regarding specific chemical agents which lead to the disruption and dissolution of the inorganic salts and organic matrices of mineralized structures; e.g., glandular secretions and various extracellular and intracellular metabolites, acids, chelators, enzymes, and combinations of chemical and physical factors.

When comparing the various mineralized structures that succumb to decalcification in biological systems, one is impressed by the broad spectrum of their chemical and physical properties, as well as by the variety of the biological organisms and biochemical agents involved in their dissolution. Rock-boring organisms can disintegrate not only relatively soft sedimentary rock, but also densely mineralized calcareous products. Boring sponges burrow not only into corals, but also into limestone and shells whether composed of calcite or of aragonite. Gastropods "drill" into the shells of bivalves as well as those of their own fellow snails. Excised gastropod boring organs can act on hard tissues other than shells and will produce etchings when the calcium phosphate crystals of human enamel and dentin are exposed to them *in vitro*. Indigenous oral microorganisms evidently are endowed with a dual capacity to produce agents which can dissolve and digest hard tissues of as contrasting composition as enamel and dentin, so as to cause tooth decay, now definitely established as being of bacterial origin. Subterranean fungi under postmortem conditions produce boring canals by a dissolution of the collagen and calcium phosphate in buried bone, ivory, cementum, and dentin, but leave dental enamel alone. Marine fungi attack the shells of bivalves (calcite) and snails (aragonite) and

have the biochemical capacity to digest the organic conchiolin shell matrix.

Vertebrate hard tissues prone to biological destruction are not entirely uniform with regard to the nature of their organic scaffolding and inorganic building blocks. Presumably there may also be different molecular bonds which bridge the two, i.e., the organic-inorganic linkage which renders the biological whole—be it shell, pearl, ivory, or bone—something far more complex (as well as more beautiful) than a simple summation of the chemical parts. The complexity of analyzing this problem has been illustrated by a number of biological systems described in this volume.

All together at least half a dozen destructive influences appear to be at work: acid demineralization, chelation, enzymatic digestion, proteolysis, molecular bond disruption of the organic-inorganic linkage, cellular ingestion, possibly phagocytosis, physical motion, and mechanical friction. A combination of two or more if not all of these mechanisms may well exert their influence at some stage of destruction within one and the same biological system. The more readily understood physical forces are at work in the case of large multicellular organisms, e.g., the twisting motion of rock-boring bivalves and the drilling action of the snail rasping holes in a shell region partially softened by decalcification. Yet anyone who has observed the cinematographic recordings of the lively process of experimental bone resorption in tissue culture will have a vivid impression that the osteoclast—aside from its complex biochemical apparatus—does in fact move around in a slow-motion "twist," rubbing its pseudopodia along the presumably softened walls of an eroding Howship's lacuna.

Though the discussion is primarily focused on the destructive aspects of hard tissue biology, it is noteworthy that a variety of systems in fact exhibit closely related constructive (biopositive) and reparative phenomena; in other words, that we are dealing with interrelated three-way processes: formation, destruction, and reformation at the cytological level; mineralization, demineralization, and remineralization at the molecular level; in short, cellular and chemical remodeling.

In coral reef remodeling the extensive "erosion" of the dying

coral skeletons by boring sponges is countered by extensive rebuilding through the calcifying powers of the living coral polyps. As rock-boring mussels channel their way into mineralized structures, the dissolved calcareous material is deposited on the walls of the burrows. In vertebrate hard tissues, redeposition of new large inorganic crystals takes place, at least at the ultrastructural level, within superficially altered tooth substance both in erosion and in caries. Such intermittent recrystallization may in part have a "reparative" significance. For example, the large crystals filling the dentinal tubules in dental erosion could possibly explain the failure of oral microorganisms to invade the dentin substance; and, similarly, in dental caries the large crystals noted in partially demineralized areas have been found to contain an exceptionally high amount of fluoride, which presumably would make such tooth substance less soluble. Moreover, when the dental enamel is exposed to a demineralizing solution, the "first order" diffusion-controlled reaction can be inhibited by deposition of protective reaction products in equilibrium with acid solutions (dicalcium phosphate and calcium fluoride on the surfaces of hydroxyapatite and fluorapatite respectively). In brief, dental erosion and caries, destructive as they are, can no longer be looked upon as entirely one-way processes, at least not from the point of view of molecular biology.

In terms of protective mechanisms there is limited knowledge regarding certain organic coatings which appear to modify the destructive processes in teeth as well as in shells, and possibly in bone. Thin salivary films which cover the tooth surfaces appear to have a significant bearing on the relative protection of the thin external layer of enamel in early caries. Furthermore, it has been suggested that dental erosion may in part be due to the absence of the protective action of such a salivary film. The mussels, whose rock-boring capacity is assisted by a calcium-dissolving secretion, have an organic protection against decalcification of the mussels' own shells, these being covered by a thick periostracal horny covering. When snails and octopuses erode the shells of oysters and abalone, it must be presumed also that a preferential shell destruction of their prey can occur only if the gastropod's own radula is protected from both mechanical and chemical action through the presence of

either some organic coating or a different crystal structure of the denticles, or both.

In addition to such potential protection of the inorganic phase against demineralization, other factors appear to control the enzymatic breakdown of the underlying organic framework. Observations on the action of collagenase suggest that the amorphous ground substance may serve as a protective coating which could modify the collagenolytic activity. The pertinence of this concept in dissolution of bones and teeth has not been fully established. The very same substance, presumably an acid mucopolysaccharide, has been thought to be involved in the process of calcification (see *Calcification in Biological Systems*). The two concepts could conceivably be reconciled, however, were one for the moment simply to suggest that the ground substance serves a stabilizing function.

When all is said and done, it will appear that the weakest link in our present fundamental understanding of the mechanisms involved in dissolution of mineralized structures relates to the specific chemical agents located in *immediate juxtaposition* to the dissolving surfaces. Whereas the living culprits of destruction generally can be identified at the "scene of the crime"—be they gastropods, mollusks, sponges, algae, fungi, osteoclasts, or bacteria—the precise microenvironments in which these biological systems operate present great difficulties in research and consequently certain differences in interpretation.

It is hoped that this volume may serve as a springboard for further research on hard tissue biology throughout the animal kingdom, especially at the relatively unexplored level of molecular biology.

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September, 1963

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1

Rock-Boring Organisms

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BEARING in mind the problems presented by this mode of life, the habit of rock boring is surprisingly widespread among marine organisms. Among plants, it is found in a variety of green, blue-green, and red algae and also in some fungi. Boring animals include certain sponges, a flatworm (Turbellaria), various sipunculid and polychaete worms, certain echinoid echinoderms, a genus of barnacles, and a diversity of gastropod and, above all, bivalve molluscs. In general these organisms are inhabitants of shallow, most often intertidal, waters. Although of wide occurrence where suitable substrates exist in temperate and tropical seas, they are undoubtedly most abundant on tropical coral reefs and within mudstones in temperate waters. Personal experience of boring organisms has been gained during the course of the Great Barrier Reef Expedition of 1928–1929 and, more recently, on the central California coast while working during 1949 and subsequently at the University of California, Berkeley, and at the Hopkins Marine Station of Stanford University at Pacific Grove.

The habit of boring is obviously not primitive. The substrate bored is either relatively soft sedimentary rock or else the calcareous product of animal secretion, notably coral skeletons and the shells of molluscs, especially the larger Bivalvia. Certain organisms, it may be noted, such as serpulid polychaetes which secrete a calcareous tube and the neogastropod Coralliophilidae, e.g. *Magilus,* settle

upon corals and extend their shells to keep pace with the growth of these. The final appearance gives a misleading impression of boring. Organisms bore either by mechanical or, if the rock be largely or in part calcareous, by chemical means. The firmer the substrate (e.g. many bivalve shells), the greater the need for at least some chemical assistance in boring.

Significance and Mode of Boring

The ability to bore invariably confers a high degree of protection, and this certainly represents the biological reason for the prevalence of the habit. Unlike the wood-boring bivalve Teredinidae (shipworms) or the crustacean *Limnoria* (gribble), which obtain much of the energy for boring from the material into which they penetrate, it is the exception for rock borers to obtain energy in this manner. Nevertheless we may conveniently begin by considering cases where boring is either certainly or possibly associated with feeding.

Some Association with Nutrition

Plants. The one certain case where energy is obtained by the borer is that of the fungi which ramify through dead or living bivalve shells, utilizing the energy present in the organic conchiolin matrix of the shell. The best-known instance is provided by the causal agent of Dutch shell disease, which formerly did great damage to European stocks of oysters, often spreading as spores to the living oyster shells from dead shells used as a settling surface or "cultch." Korringa (1952) has described the life history; although oysters die if the shells become heavily infected, this is due to reaction by the molluscan tissues. The fungus itself never penetrates the tissues. Presumably boring is along the areas of conchiolin, although some actual penetration of the calcified regions by either mechanical or, more probably, chemical means may well be necessary.

A variety of filamentous green, blue-green, and also some red algae penetrate into calcareous rock or shells, either deeply or superficially. Blue-green algae are abundant between tidal levels on coral reefs, causing a softening which may be due to the action of CO_2 or other

acids produced in metabolism (Newhouse, 1954; Newell, 1955, 1956). Less easy to understand is the boring activity of the green algae which penetrate into living coral rock, where they form a green zone up to 3 mm within the skeleton. They may also occur in dead coral fragments. The commonest genus appears to be *Achyla,* but others are listed by Utseumy (1942). The little that is known about the manner of boring indicates that this is due to the combined action of acid production and growth pressure. There is also the suggestion that entrance may be associated with the presence of nutritive matter, presumably derived from the small organic content within the skeleton (Duerden, 1902, 1905). Odum and Odum (1955) have recently suggested that there may be some symbiotic relation between the corals and these algae, but, in the absence of any experimental evidence, this is difficult to accept.

A full understanding of the mode of boring and of the source of material needed for protein synthesis in these algae is badly needed. But the problems are complex and investigation is difficult.*

Animals. *Possible direct association.* Certain animals which bore into coral rock may possibly gain all or a significant part of their food from the boring algae and the almost equally common and widely ramifying sponges (described later). This could apply to the sipunculid worms, such as the numerous species of *Aspidosiphon,* which commonly bore into coral rock. The small cuticular plates present in the skin of sand-burrowing sipunculids are here fused to form massive shields with which the animal bores. Since the primitive habit is burrowing into sand, which is swallowed in great quantities with digestion of the small amount of contained organic matter, change to a rock-boring habit presents no major problem. It is certainly possible that the rock into which the worms bore may contain as much organic matter, in the form of boring plants and sponges, as the sand.

What applies to these sipunculids may also, although less probably, apply to some of the coral-boring polychaetes. Gardiner (1903) has listed the more important families. According to Crossland

* Since the above was written, knowledge has been significantly advanced by Wainwright (1963).

(quoted by Gardiner, 1931), the largest Indo-Pacific species "are *Leocrates* (*Eunice*) *siciliensis, Lysidice collaris* and some of the Cirratulidae. The former two have enlarged gouge-shaped lower jaws, with hard, though calcareous, cutting edges, which are obviously their boring tools, but the latter have soft bodies and no conspicuous hard parts at all." Unlike that of the sipunculids, the mode of feeding of related nonboring species does not indicate that they may get food by boring. Like the temperate-water *Polydora* (described later), they may bore solely for protection. Study of these forms in life would solve this problem.

The turbellarian flatworm *Pseudostylochus ostreophagus,* which is a native of Japanese waters but was introduced into Puget Sound with consignments of Japanese oysters (*Crassostrea gigas*), attacks the oysters when young, making a keyhole-shaped opening in the shell and then eating the contained animal. The process of boring is unknown, but in the absence of jaws or any other hard structure must involve chemical means. Certain carnivorous Gastropoda, notably the prosobranch Muricidae and Naticidae, bore through the firm calcareous shells of their prey, usually bivalves, by the mechanical action of the radula assisted by acid secretion from an accessory boring organ (Carriker, 1961). Later the flesh of the prey is rasped out by means of the radula. Full description of the processes involved is provided by Carriker and his co-workers in chapter 3 of this symposium.

Indirect association. We are here concerned with animals which scrape the surface of calcareous rock to obtain the algae which grow upon it or bore superficially into it. This is true of species of largely intertidal prosobranch Gastropoda, of such genera as *Nerita* and *Turbo,* which cut into the softened rock surface with the broad radula and assist in the formation of the characteristic intertidal "nick" along the landward margin of coral reefs (Doty and Morrison, 1954; Newell, 1955, 1956; Newell and Imbrie, 1955). But in no real sense can these gastropods be said to bore.

Much the same habit exists in a variety of echinoid echinoderms (sea urchins) which excavate new borings or enlarge old ones by means of the teeth and spines. This is purely for protection, primarily against wave action but to some extent on exposed shores against

desiccation, and occurs only when such dangers exist. It is a purely facultative habit and is found in various intertidal echinoids. Umbgrove (1947) considered *Echinometra mathaei* to be a major agent of erosion in East Indian coral reefs.

Only when the borings become deep and finally flask-shaped are the animals imprisoned within them. Usually they are able to leave the "boring" and forage on the surrounding rock surface. Food consists largely of encrusting algae, and probably sufficient fragments are carried in by water movements to support imprisoned animals. But any algae growing on or within the rock in the borings will be a source of food. There is no question here of chemical action, indeed the rock may not be calcareous, and boring is to a large extent fortuitous. The whole matter has been most thoroughly reviewed and discussed by Otter (1932).

No Association with Nutrition

Sponges. A variety of sponges, most notably and ubiquitously the numerous species of the genus *Cliona*, bore into calcareous rocks, into coral skeletons, and into mollusc shells. *Cliona celata*, which bores into calcareous structures, especially oyster shells, in European waters is probably the best-known species. The larvae settle from the plankton and the developing sponge quickly penetrates the shell, forming extensive galleries so that the whole structure becomes friable and crumbles away. There can be no question here of the sponge's gaining food in this way; like all sponges, it feeds on nanoplankton drawn into the body in currents created by the flagella. It does not necessarily bore, indeed this would appear to depend on the nature of the substrate on which the larvae settle. The method of boring remains obscure; the siliceous spicules could assist mechanically, but, in view of the calcareous substrate, acid could obviously be used. Growth processes could also assist, as suggested in the case of algae. Indeed, as noted by Gardiner (1903), the algal *Achyla* and *Cliona* resemble each other in their mode of growth; Gardiner adds that "their ramifications are most delicate, imperceptible to the unaided eye, and wander all over the coral skeleton. In *Pocillopora* their terminal filaments extend so close to the ends of the branches that only the very thinnest layer of corallum separates the polyp

tissues from them. When a branch of this genus is decalcified and the polyp layer carefully stripped off, either of these boring organisms will be seen to have formed a close-meshed network, showing accurately the shape of the original branch with all its twigs." Gardiner considered that neither of these organisms bores into anything but newly secreted coral skeletons. As noted above, they could provide food for boring sipunculids and, less probably, polychaetes. The different, much more finely ramifying, type of growth within coral as compared with bivalve skeletons may well be related to differences in the skeleton, possibly in the organic content. Goreau and Hartman add greatly to our knowledge of the boring of sponges into corals in their contribution to this symposium (chapter 2).

Polychaetes. The best-known polychaete rock borers, always apparently boring into calcareous substrates, are species of *Polydora,* which may be serious pests of oysters in many parts of the world. These small worms initially form mucous tubes to which mud adheres. They tend to back into crevices and can then bore if the substrate is suitable. They do so with the body bent double into a U, the arms of which are separated by a partition of mud and debris consolidated in mucus. Boring must be in part, if not wholly, mechanical, possibly by the agency of enlarged dorsal setae on the fifth setigerous segment. Since the substrate is apparently always calcareous, boring could be assisted by some initial chemical action, although there is no evidence of any glands on the surface of the body. Other species inhabit crevices in shale and sandstone. There seems to be some doubt as to whether these ever bore; if not, it could be because the rock is harder, not because it is noncalcareous.

Cirripedes. The attached habit of barnacles has led, following fixation to the body of animals, to various degrees of commensalism culminating in parasitism. It has also, most notably in the genus *Lithotrya,* led to penetration of the rock on which the cypris larvae settle. Species of this genus are among the commonest borers on both Indo-Pacific and Atlantic coral reefs (Cannon, 1935; Seymour Sewell, 1926). The chitinous covering of the contractile peduncle contains numerous "nail-like bodies, the head of each nail being

originally enclosed in a small calcareous head" (Seymour Sewell, 1926). By this means the animal excavates a cavity into which the contracted peduncle fits, with the opercular plates just flush with the surface, so that even when common these barnacles are initially most difficult to detect. The peduncle is attached to the boring by way of a calcareous basal plate a little distance from the tip on the carinal side (Fig. 1). This supplies the necessary purchase for bor-

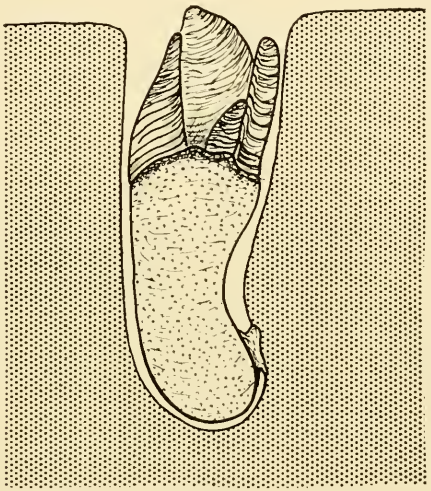

FIG. 1. *Lithotrya nicobarica,* probable appearance when withdrawn within boring. (Modified after Seymour Sewell, 1926.)

ing, although, of course, the position of attachment must continually alter during growth (the same problem arises in the case of the boring bivalve *Tridacna crocea,* as described below). The process of boring must be purely mechanical. Like other barnacles, *Lithotrya* feeds on zooplankton organisms by means of its setous thoracic appendages.

BIVALVE MOLLUSCS. The basic structure of these animals ideally fits them for boring. Enclosure of the entire body by the mantle and shell has led to loss of the head and enlargement of the gills, which have assumed the added function of collecting food consisting of phytoplankton. Except in a few primitive and some specialized

groups, both intake and extrusion of the essential water current take place at the posterior end. Hence when these animals have succeeded in penetrating into rock they have achieved maximum protection while continuing to feed by ciliary currents in the normal manner. There is no question of any of them obtaining anything of food value from the rock. The shell valves are always the prime agents of boring, although the manner in which they operate to this end differs greatly. Only in certain members of one superfamily (vii below) is the mechanical action of the shell valves undoubtedly assisted by chemical means.

Origin and Nature of Boring in the Bivalvia

So well are these animals basically fitted for boring that members of no less than seven superfamilies of the Bivalvia have, independently of one another, taken to this mode of life. It is interesting also that they have arrived at this final habit by one of two routes.

Primitively the Bivalvia are members of the *infauna,* i.e. they live within soft substrates through or down into which they burrow by means of the initially hatchet-shaped foot terminally dilated by blood pressure. By further development of the habit of deep burrowing they have come to penetrate stiffer and stiffer substrates, finally becoming true borers. This is true of some or all of the members of the first two superfamilies (i and ii) described below.

Certain Bivalvia, e.g. marine mussels, have secondarily become permanently attached in adult life to a hard substrate, i.e. they have become members of the *epifauna.* For reasons given elsewhere (Yonge, 1962), this is probably due to retention into adult life of the mechanism of attachment by byssal threads. This probably was originally a larval structure concerned solely with temporary attachment of the newly settled larva, and this certainly constitutes its sole function in the more primitive infaunal bivalves. The epifaunal habit brings the bivalves into intimate association with rock, and this is the certain, or very probable, route whereby the boring habit has been reached in superfamilies iii to vii.

The degree of specialization exhibited within the different groups

of rock-boring bivalves varies very greatly, as briefly outlined below.

Infaunal Origin

i. Superfamily Myacea. *Platyodon cancellatus,* the sole boring species (and genus), somewhat resembles the soft-shelled clam, *Mya arenaria,* to which it is closely related. It is confined to the California coast between 33° and 38° north latitude, where it occurs between tidemarks, boring in relatively soft mudstones (Yonge, 1951). It bores mechanically by means of the shell valves, which are ridged and much eroded in the umbonal regions. Compared with *Mya,* the valves are more convex (see Fig. 2), and the con-

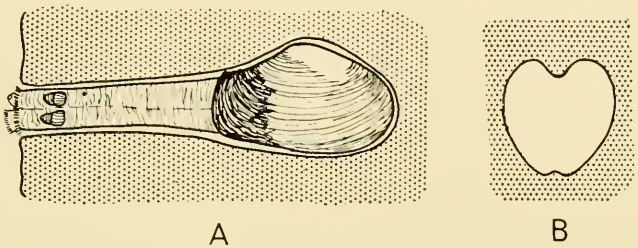

Fig. 2. *Platyodon cancellatus.* A, animal in boring, showing erosion of shell around umbones, also periostracal "scales" near tip of siphons; B, section through boring in umbonal region. (After Yonge, 1951.)

densed ligament or chondrophore is displaced somewhat posteriorly. The horny periostracum which covers the long siphons is thickened near the tip to form four "scales," which represent the only obvious adaptation for boring.

Platyodon bores directly into the rock, i.e. without twisting. As a result the boring is not rounded in cross section, but, in the region occupied by the shell, has "dorsal" and "ventral" ridges, the former, which lies between the rounded umbones, being very pronounced (Fig. 2B). Boring is the result of lateral pressure by the valves due to taking of water into the mantle cavity, followed by closure and withdrawal of the siphons. Such pressure can be only slightly increased by the opening thrust of the somewhat reduced ligament,

but there may be some rocking on the median fulcrum of the reduced hinge and ligament by reason of uneven contractions of the two adductor muscles. The valves gape both anteriorly and posteriorly. Judging by the greater erosion, most work may be done by the umbonal (but also oldest) regions of the shell. The siphonal extension of the boring is certainly enlarged by means of the four periostracal scales. The reduced foot has no apparent function in boring.

ii. Superfamily Adesmacea. All members of this large superfamily are borers, the more specialized Xylophaginidae and Teredinidae living in wood, the majority of the Pholadidae boring into stiff clay or rock. Species of *Zirphaea* which burrow into very heavy clay probably indicate the manner in which the boring habit has evolved. But these are now the only members of the superfamily which do not bore. There is in all, including *Zirphaea,* great modification of structure (see Lloyd, 1897; Purchon, 1955*a*) in relation to the purely mechanical process of boring. The valves tend to be elongate, to bear rows of teeth or ridges, and to be very convex. By a reduction of the mantle isthmus which runs between the two lobes of the mantle, the ligament is either much reduced or completely lost, i.e. there is separation of the mantle lobes and shell valves. Hinge teeth disappear and are replaced by dorsal articulating processes on which the shell valves, which gape anteriorly and posteriorly, rock. In certain genera (e.g. *Pholadidea,* as shown in Fig. 3, *VA*) there are secondary ventral articulatory surfaces. Both adductors migrate dorsally so that they come to lie in the same line as the dorsal articulating surface. In the anterior adductor this involves a rolling upward of the anterodorsal region of the valves. By cross fusion of the inner muscular lobe of the mantle margin, a third, mid-ventral, adductor muscle is formed. Following the alternate contraction of the anterior and posterior adductors, the shell valves rock on the fulcrum of the dorsal articulation (and ventral articulation where this is present).

Attachment to the head of the boring, essential for effective boring action, is by way of the foot modified to form a sucker. This projects forward through a wide anteroventral gape between the

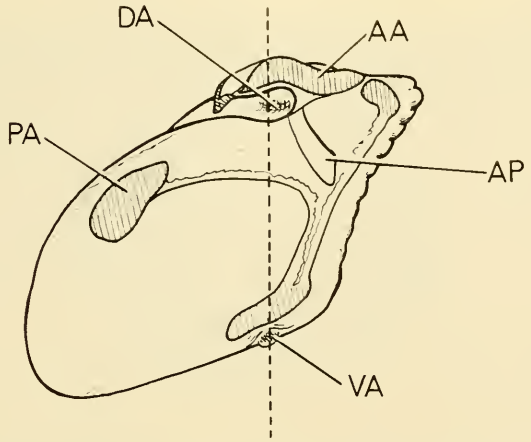

FIG. 3. *Pholadidea penita*, left valve, showing dorsal (*DA*) and ventral (*VA*) articulations, anterior (*AA*) and posterior (*PA*) adductors, and apophysis (*AP*) to which retractor muscles of foot are attached. Foot protrudes through pedal gape anteroventrally. Shell valves rock on axis of motion indicated by broken line. (After Lloyd, 1897.)

valves. The changed position, and function, of the foot involves change in disposition of the pedal muscles. These are now attached to a pair of bladelike shell processes which extend ventrally from within the hinge region. These apophyses (Fig. 3, *AP*) are found only in the Adesmacea.

During boring the animal rotates, the attaching foot moving first in one direction, then in the other. Thus a smooth boring is cut by the rocking of the valves on the median fulcrum. The effective abrasive action is performed by the anterior halves of the valves, which are pulled apart by the action of the posterior adductors. In section the boring is round with no trace of dorsal or ventral ridges.

In the most specialized of the adesmacean rock borers, *Pholadidea* and *Parapholas,* the animal excavates a boring of a definite size and then the foot with its musculature atrophies and the anteroventral gape is filled in by inward growth of the mantle margins, which secrete a thin callum over the entire surface. This is not true of *Zirphaea, Pholas,* or *Barnea.*

Apart from the stiff clay in which species of *Zirphaea* occur, to depths of up to 50 cm, the pholads bore into a wide variety of sub-

strates including slate, shales, sandstone, chalk, marl, and even peat and submerged wood. *Pholadidea penita* has been found in concrete casings. In all, the fused siphons are long, the basal half, which is encased in a protective periostracum, representing posterior extension of the mantle cavity and housing the posterior region of the gills. The pholads do not appear to bore significantly into limestone and, although usually the most important rock borers in other regions, are certainly largely absent from coral reefs.

Epifaunal Origin

iii. Superfamily Veneracea. Family Petricolidae. The typical venerid burrows very superficially, but in certain species byssal attachment persists, and these forms inhabit rock crevices. In the Petricolidae this habit is taken further and the animals bore into the rock. There is an interesting range of specialization within the genus *Petricola.* Thus in *P. carditoides* (Fig. 4A), common in holes in rocks on the California coast, the animal does no more than enlarge these as it grows; the process is entirely mechanical by means of the shell valves, water pressure being possibly materially assisted by the opening thrust of the long and unmodified ligament. The byssus is not retained in boring. Great secretion of mucus, both in the mantle cavity and in the siphonal embayment, is associated with this mode of life (Yonge, 1958). The shell may be almost globular or elongate; it is often very irregular in form. At the other extreme comes *P. pholadiformis* (Fig. 4B)—so named because of the superficial resemblance of its elongate, ridged shell to that of a pholad—which bores with great efficiency (Purchon, 1955*b*). Species of *Petricola* bore into a variety of substrates, from stiff mud, mudstone, and shales to limestone. *Petricola lapicida,* with boring capacities intermediate between those of *P. carditoides* and *P. pholadiformis,* is not uncommon in beach rock, coral boulders, etc., on the Great Barrier Reef (Otter, 1937).

iv. Superfamily Saxicavacea. Genus *Hiatella* (*Saxicava*). Species of this widely distributed genus may either live in rock crevices attached by byssus threads or bore. It is not clear whether certain species always bore and others always "nestle"; indeed, there is

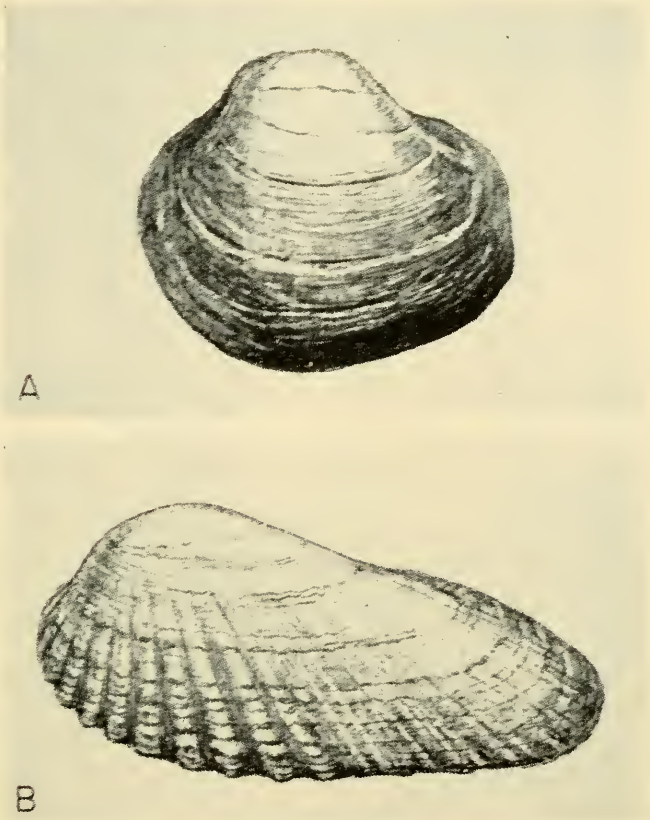

FIG. 4. A, *Petricola carditoides;* B, *P. pholadiformis;* showing greater specialization of the shell for boring in the latter. (From Yonge, 1958.)

evidence that the boring habit is facultative and not obligatory. In his account of *Hiatella gallicana* and *H. arctica,* Hunter (1949) states that the habit of the adult is determined by the nature of the substrate on which the larvae settle: if it be the surface of a soft homogeneous rock they will bore, if it be a hard but creviced rock surface they will become byssally attached. The byssus threads (but not the gland) are lost in animals which bore, the process being entirely mechanical (the valves becoming much eroded) by means of water pressure forcing the shell valves apart (Fig. 5). Hunter points out that "the boring movements evolved with little

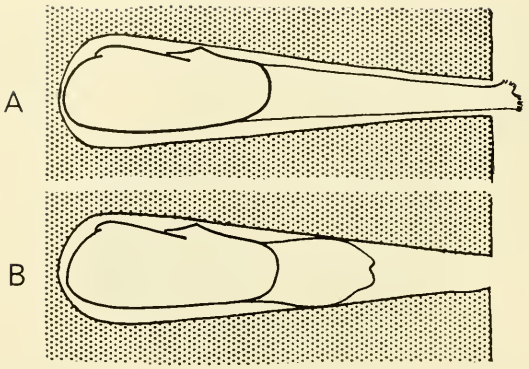

FIG. 5. *Hiatella* (*Saxicava*) sp.: A, showing full extension of siphons for feeding and respiration; B, contraction of siphons (openings closed) ready for boring, valves forced apart by water pressure, and thickened siphons gripping sides of boring. (After Hunter, 1949.)

modification from the protective reactions of non-boring animals."

Hiatella is confined to temperate seas in both the Atlantic and the Pacific. It bores into comparatively soft rocks, i.e. muddy limestones, mudstones, sandstones, calcite, and chalk (Hunter, 1949). Although all these rocks are broken down by acid, i.e. the binding cement is acid soluble, there is no evidence that boring is aided chemically. The form of the shell varies so greatly, especially in boring individuals, that specific identification is unusually difficult. However, species of *Hiatella* are most efficient borers, cutting deep borings which are round in cross section.

v. SUPERFAMILY GASTROCHAENACEA. GENUS *Rocellaria* (*Gastrochaena*). Members of this genus are highly specialized borers of warm-temperate and tropical seas, always boring into calcareous substrates. They are common in Indo-Pacific coral reefs (Otter, 1937). As shown in Fig. 6, there is some superficial resemblance to the Pholadidae, notably in the similar possession of a suckerlike foot projecting through a wide anteroventral gape and also of small internal shell ridges to which the pedal muscles are attached (Purchon, 1954). In other respects, however, there are major differences, most obviously in the ligament, which is here very long, so that the valves cannot possibly operate in boring as do those of

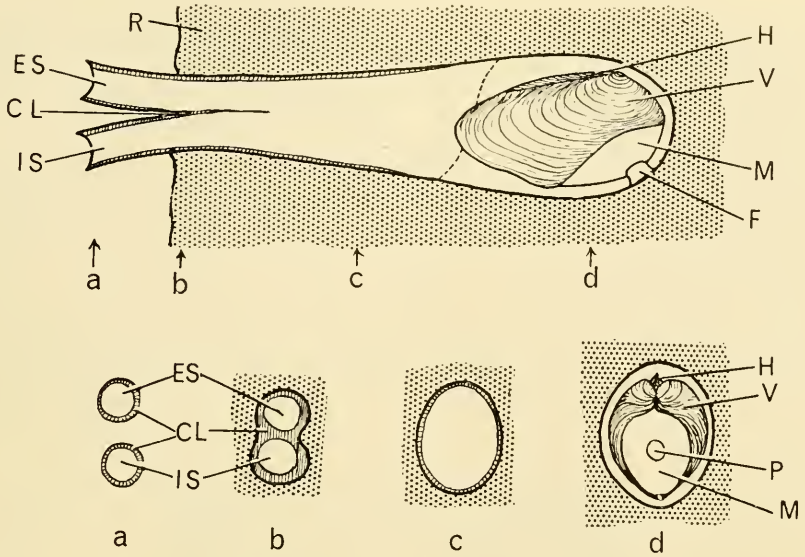

FIG. 6. *Rocellaria* (*Gastrochaena*) *cuneiformis*. *Above*, animal *in situ* in boring; *below*, transverse sections in regions *a* to *d*. *CL*, calcareous lining secreted by walls of siphons (not shown) and forming projecting exhalant (*ES*) and inhalant (*IS*) siphonal tubes; *F*, suckerlike foot; *H*, hinge region; *M*, mantle exposed in anterior pedal gape; *P*, pedal opening; *R*, rock; *V*, shell valve. Broken line denotes inner limit of secreted calcareous lining of boring. (After Otter, 1937.)

the Pholadidae, but must abrade as a result of water pressure aided by the opening thrust of the long ligament. A striking feature of *Rocellaria* (indeed of the entire superfamily) is the capacity of the siphonal tissues to secrete calcium carbonate (Sluiter, 1890), so lining the boring and extending this beyond the rock surface into two tubes formed by the separated ends of the siphons (Fig. 6, *ES*, *IS*). The boring is oval in section (Fig. 6 *c*, *d*), indicating, together with the fixed position of the siphonal tubes, that the animal bores directly into the rock. The borings appear to be much longer than even the long siphons, and possibly, unlike the Pholadidae, these animals can move backward and forward within the boring. A byssus gland persists, and occasionally threads are secreted. This fact, together with the habits of related genera, indicates that here also boring has followed an initial epifaunal habit.

vi. Superfamily Cardiacea. Family Tridacnidae. *Tridacna crocea.* This species, confined to the mid-tropics in the Indo-Pacific, where it is often extremely abundant, boring into coral boulders, is perhaps the most unexpected of bivalve borers. It belongs to a family which includes the largest bivalve ever evolved, *Tridacna deresa,* the giant clam. All species in the family begin life attached by byssus threads. In the larger ones the byssus and the shell gape through which it projects disappear and the animals finally maintain themselves by their unaided weight. But in *T. crocea* the byssus, and the gape through which it emerges, become larger with age, and at the same time the animal proceeds to bore *downward* into the calcareous rock (Fig. 7). This is a consequence of the

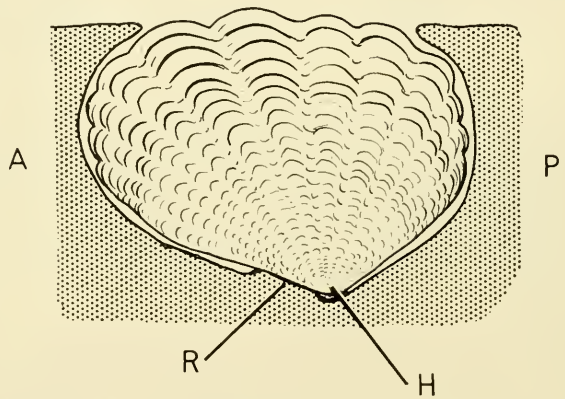

Fig. 7. *Tridacna crocea,* viewed, from left side, *in situ* within boring in coral rock, shown in longitudinal section. Animal lies with hinge (*H*) undermost, massive byssus (obscured by shell) attached to pillar (*R*) anterior to this. *A, P,* anterior, posterior. (After Yonge, 1936.)

unique form of these animals. The shell valves have changed their position in relation to the enclosed body so that the hinge comes to lie beside the byssal gape on the underside of the body. The result is that the siphonal tissues (normally at the posterior end) extend along the upper, i.e. dorsal, surface. They also extend laterally, forming a broad band of brilliantly pigmented tissues facing upward to the light. Within these tissues are contained countless mil-

lions of unicellular algae or zooxanthellae. The Tridacnidae literally "farm" plants in these illuminated tissues, and this is the biological explanation for the remarkable change in form (Yonge, 1936, 1953). The presence of the hinge, instead of the free margin of the valves, on the underside alongside the byssus has made boring possible. Running between the byssus and the inner surface of the valves are extremely powerful pedal (or byssal) retractor muscles. Alternate contraction of these muscles causes the animal to rock in the longitudinal axis on the fulcrum of the byssus. These rocking movements are totally distinct from those of the Pholadidae, which are in the median transverse plane and caused by alternate contractions of the two adductors.

Entry in *T. crocea* is vertical but to some extent diagonal to allow for undercutting of the byssal attachment on the one side while new threads are formed on the other. The byssus is attached to a pillar (Fig. 7, *R*) at the base of the boring which fits into the byssal gape on the underside of the shell. The thick-ridged shell valves are always greatly eroded where they bear against the surface of the rock. After they begin to bore (when about 1.4 cm long), the animals must quickly penetrate to the depth of the shell valves, which remain flush with the surface during growth to a final length of some 10 cm, when the boring will be about 7 cm deep. The animal is imprisoned within the boring, the length of the shell being greater than that of the opening (see Fig. 7). Except when withdrawn during exposure at low tide, the hypertrophied siphonal tissues spread out over the surface of the rock, completely covering both the margins of the valves and the sides of the boring. The high temperature at which these animals spawn, about 30°C, confines them to shallow water in the mid-tropics.

vii. Superfamily Mytilacea. Family Mytilidae. This large family, which includes the common mussels, contains two important genera of rock borers, *Botula* and *Lithophaga*. Both are byssally attached throughout life, but whereas the former bores only into soft, usually noncalcareous, rocks such as mudstones and shales, the latter is confined to calcareous rocks—limestones, calcareous shales,

carbonate-cemented sandstones, shells of other molluses, and above all coral rock—and boring is certainly assisted by initial chemical softening of the rock (Yonge, 1955).

In both genera the shell is elongated and almost tubular, a modification of the somewhat triangular shell form of the typical mussel. This form, brought about by modification of the components of shell growth (see Yonge, 1955), enables them to penetrate rock with great efficiency. In *Botula* (Fig. 8) a massive byssus is secreted.

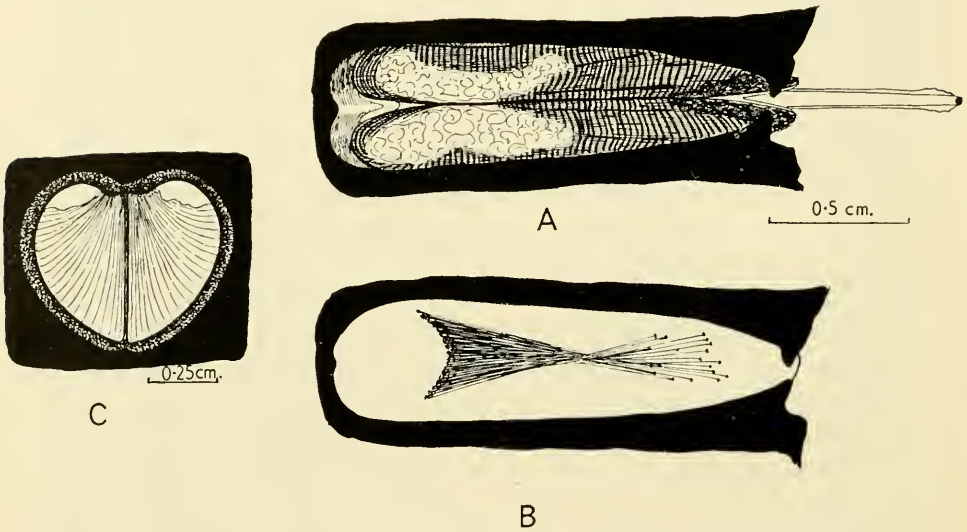

Fig. 8. *Botula falcata*. A, boring exposed to show dorsal side of animal, siphons extended and animal withdrawn from head of boring; erosion of shell on either side of hinge region shown. B, boring opened to reveal attachment to "floor" of boring of byssal threads in large anterior and smaller posterior groups, former pulled against when animal bores. C, boring opened at head end. (From Yonge, 1955.)

So attached to the floor of the boring, contraction of the posterior byssal retractor muscles drives the anterior end of the shell forward against the head of the boring. There can be no question here of water pressure within the mantle cavity because there are no true siphons, but the ligament is long and its opening thrust powerful enough to cause widening of the boring. Although the shell has a thick covering of brown periostracum, this and the underlying

calcareous layer are invariably deeply eroded around the umbones (Fig. 8A). Boring is straight into the rock, with a pronounced dorsal and a smaller ventral ridge (Fig. 8C). *B. californiensis* and *B. falcata* are both common borers, largely between tidemarks, in soft rocks along the coasts of central California where they were examined (Yonge, 1955).

Lithophaga (*Lithodomus*) *lithophaga*, the date mussel of the Mediterranean, has been known since classic times. There are many other species in warm-temperate and tropical seas, and they are particularly common on coral reefs, five species occurring on Low Isles, Great Barrier Reef (Otter, 1937). Compared with *Botula*, the shell is more completely rounded in section and is everywhere covered by a particularly thick and dark-colored periostracum. This is *never eroded*. In most species of *Lithophaga* anterior and posterior areas of the shell are covered with a granular calcareous deposit (Fig. 9). Although byssal attachment persists throughout

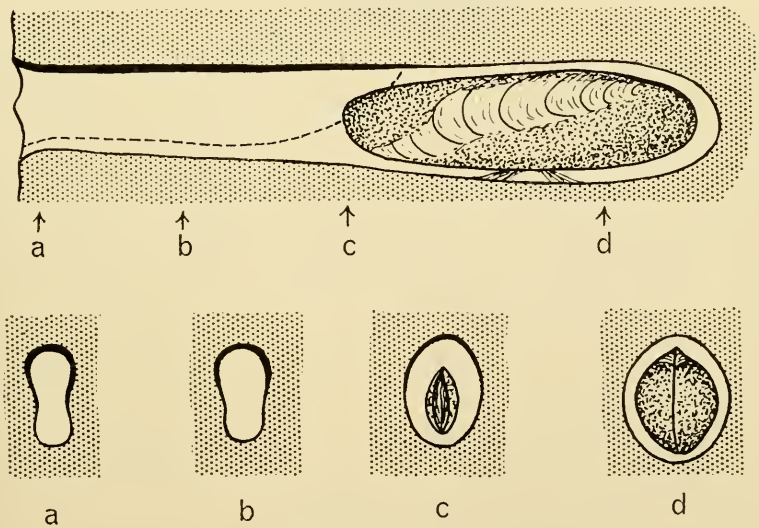

Fig. 9. *Lithophaga cumingiana*. *Above*, animal *in situ*, in boring; *below*, transverse sections in regions *a* to *d*. Small extent of byssal attachment shown, also adherent calcareous debris over anterior and posterior regions of shell (but *no* erosion). Calcareous lining of boring (as in *Rocellaria*) secreted by walls of siphons, indicated in section by thick line, marginally (ventrally and anteriorly) by broken line. (After Otter, 1937.)

life, the number of threads and strength of attachment are far less than in *Botula* (Yonge, 1955). This condition is correlated with an initial chemical softening of the rock. The mantle tissues protrude from between the valves anteriorly, spreading out laterally over their surfaces. When the animal is pushed against the head of the boring, these tissues are applied to the surface of the rock (Fig. 10).

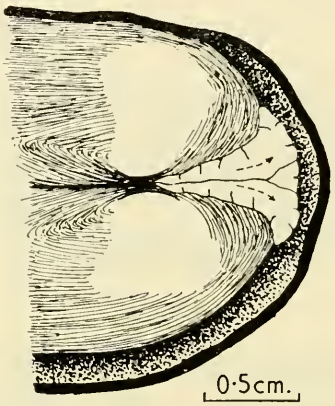

Fig. 10. *Lithophaga plumula*, boring opened from above to show anterior end of shell with mantle tissues protruded against head of boring. Arrows show direction of ciliary currents. (From Yonge, 1955.)

Although the presence of acid, as such, cannot be demonstrated, softening and dissolution of calcareous rock against which the tissues are applied has been demonstrated by Kühnelt (1930), Yonge (1955), and Hodgkin (1962). The last named has also demonstrated experimentally that, when placed in a suitable-sized bore hole in mudstone, *L. plumula* is unable to enlarge it, although it can certainly rotate within it. It is quite certain that *Lithophaga* cannot bore without some initial chemical softening of the rock. Its own shell is protected against chemical action by the thick, and invariably unbroken, layer of periostracum.

The glands responsible for the softening, probably acid-containing, secretion are situated within the middle mantle fold immediately anterior and posterior to the ligament. Similar glands also occur in *Botula* and in all Mytilidae. In these mussels they secrete mucus, which entangles particles that fall on these regions (es-

pecially posteriorly) when the valves are open. The anterior glands are large only in *Lithophaga*, where alone the tissues in which they are situated can be protruded. The granular layer on both anterior and posterior shell surfaces in *Lithophaga* and over posterior regions only in *Botula* consists of calcareous fragments and other debris (only the latter in *Botula*) which are consolidated in mucus secreted by these glands (Yonge, 1955). The presence of acid in the anterior glands—and probably also in the posterior glands, which may assist in the widening of the boring—is confined to *Lithophaga*.

Some species of *Lithophaga* rotate in the boring, e.g. *L. plumula* (Yonge, 1955); others do not, e.g. *L. cumingiana* (Otter, 1937). In the majority of species—there is some degree of variation within the genus—the posterior region of the boring has a secreted calcareous lining, resembling that described in *Rocellaria* although not so well developed. This is produced by the walls of the siphons, here consisting of the inner lobe only of the mantle margin, the inhalant siphon being produced by apposition, not fusion, of the ventral margins. In certain species, e.g. *L. plumula*, the siphons may also be responsible for adventitious posterior extension of the shell valves.

Although *Botula* and *Lithophaga* doubtless descend from common ancestors, conditions in the former, exclusively mechanical, borers foreshadow those in the latter. With the development of the means of chemical softening of the rock, *Lithophaga* has lost full efficiency for mechanical boring but has exploited to the full the added powers of boring into calcareous rocks of various kinds.

Discussion and Summary

Although, as noted in the introduction, the habit of rock boring is unexpectedly widespread, this brief review indicates how many of the borers are not specifically adapted for this mode of life. In many cases boring is facultative, not obligatory. The condition in the plant borers is somewhat obscure, but among the animals boring is *not* obligatory in the sponge *Cliona*, in the echinoids, in *Polydora*, or, among the bivalves, in *Hiatella* and the less specialized species of *Petricola*. All these animals are members of the epifauna, with

a basic sessile or nestling habit which does, where conditions are suitable, lead to boring.

Really specialized obligatory borers include certain sipunculids and polychaetes, the carnivorous shell borers including the turbellarian *Pseudostylachus* and the prosobranch gastropods (relying wholly or in part on chemical agencies), the cirriped *Lithotrya*, and most of the bivalve borers. In the last-named group, where we have fuller information than about any other group apart from the carnivorous gastropods, boring is primarily mechanical by means of the valves. The majority grind by lateral and forward movements caused by pressure of water in the mantle cavity aided to greater or lesser extent by the opening thrust of the ligament. In the highly specialized Adesmacea, boring is due to the rocking action of the valves on the median fulcrum of the hinge region caused by the alternate contraction of the anterior and posterior adductor muscles. The ligament is greatly reduced or lost. In the boring Tridacnidae, where the ligament is not reduced, the animal grinds its way downward by the alternate contraction of the pedal (or byssal) retractor muscles. This causes rocking in the longitudinal plane on the fulcrum of the massive byssal attachment. In the boring Mytilidae, contraction of the posterior byssal muscles causes the anterior end of the shell to bear against the head of the boring, while lateral widening must be due to the opening thrust of the powerful, secondarily extended ligament. But in *Lithophaga*, confined to calcareous rocks, the means of mechanical boring are reduced, with accompanying modification of preexisting mantle glands to supply the means for chemical softening of the rock. But, as with other boring organisms, apart from the carnivorous gastropods, we lack knowledge about the precise manner in which the rock is softened although this presumably involves acid production.

Protection has been revealed as the major biological advantage of rock boring. Where borers are numerous, notably on coral reefs, they may be major agents of erosion. Reefs are maintained only by the exceptional powers of calcification possessed by hermatypic (reef-building) corals. Fungi, sponges, flatworms, polychaetes, and drills are all major pests of oysters. On the other hand, unlike the

wood borers, rock borers do little damage to structures erected in sea water, which are usually too hard for them to penetrate.

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2

Boring Sponges as Controlling Factors in the Formation and Maintenance of Coral Reefs

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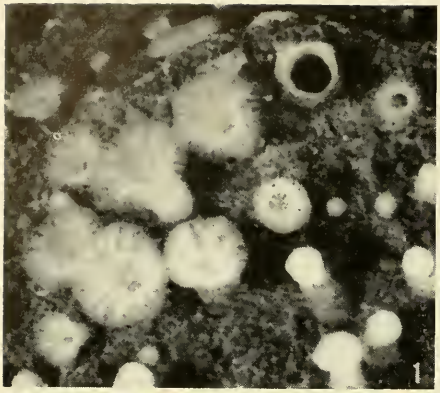
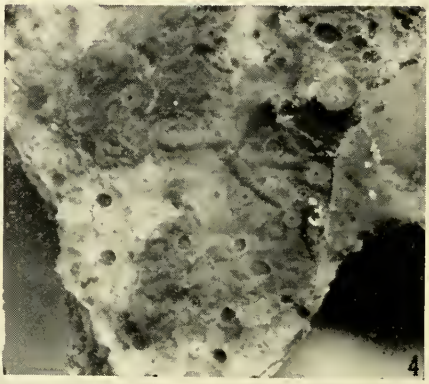
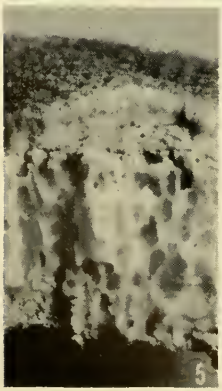
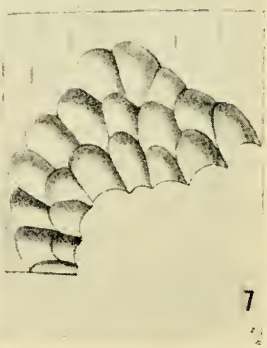
TROPICAL reef communities harbor a diverse biota of indwelling plants and animals which destroy corals, shells, and limestone (Otter, 1937). Verrill and Smith (1873) suggested that the breakdown of insoluble skeletal carbonates by marine borers is a significant factor in the calcium balance of the seas. Gardiner (1903, 1931) stated that boring algae, mollusks, and worms play important roles in the breakdown and erosion of atoll reefs, whereas Yonge (1930) believed the most obvious agents of reef destruction to be the bivalve mollusks. Ginsburg (1957) affirmed that the weakening of reefs by organic action makes them more susceptible to wave erosion, and cited boring sponges as examples.

Until the present, the destruction of reefs by burrowing organisms has been studied only in extreme shallow-water environments where the effects of boring are masked by rapid biological calcification and energetic wave attrition. However, a series of recent investigations carried out in Jamaica on the coral communities of the steep









FIGURES 1 TO 7

fore-reef slope habitat at depths between 30 and 70 meters drew our attention to the existence of major undescribed erosional features which are determined by the activity of boring sponges under conditions where $CaCO_3$ deposition in corals is not so fast as at the surface and the effect of wave turbulence is normally negligible. In such localities the consequences of sponge boring go far beyond the mere hollowing out of a few cavities: our observations show that the ecology of the reef coral population, the movement of reef sediments, and the modeling of the reef edge are all strongly influenced.

Nassonow's important work (1883) showing that clionid sponges produce fine calcareous debris in the course of their boring activities has been generally overlooked, and more recent authors (Revelle and Fairbridge, 1957; Ginsburg, 1957; Cloud, 1959) assert that sponges bore by chemical action and imply that all the excavated carbonate is removed by solution. The observations of Nassonow and others suggest, however, that the burrowing sponges may be of major significance in the reduction of reef limestone to very fine detritus.

FIG. 1. Incurrent and excurrent papillae of boring stage of *Cliona celata* Grant. Oscule in upper right. (× 3.6.) (From Hartman, 1958.)

FIG. 2. Massive, free-living stage of *Cliona celata* Grant. (× 0.36.) (From Hartman, 1958.)

FIG. 3. Broken edge of a colony of the coral *Agaricia* showing extensive excavation by *Cliona lampa* de Laubenfels. (× 0.9.) 38 meters, Maria Buena Bay, Jamaica. (Photo by T. F. Goreau.)

FIG. 4. Surface of colony of *Agaricia* showing incurrent and excurrent papillae of *Cliona lampa* de Laubenfels. An expanded incurrent papilla may be seen in the upper right; papillae with central holes are excurrent. (× 1.2.) 38 meters, Maria Buena Bay, Jamaica. (Photo by T. F. Goreau.)

FIG. 5. Broken edge of a colony of *Montastrea annularis* (E. and S.) excavated by a clionid. Beneath the coral polyp layer at the top is a layer of boring algae, seen as a dark line. The boring sponge has died and has left extensive galleries in the coral. (× 0.9.) 38 meters, Maria Buena Bay, Jamaica. (Photo by T. F. Goreau.)

FIG. 6. Larva of *Cliona* spreading out over a fragment of oyster shell. Lines etched into the calcareous matter are clearly seen. (From Nassonow, 1883.)

FIG. 7. Pattern of depressions left in an oyster shell after the removal of calcareous chips by a clionid. (From Nassonow, 1883.)

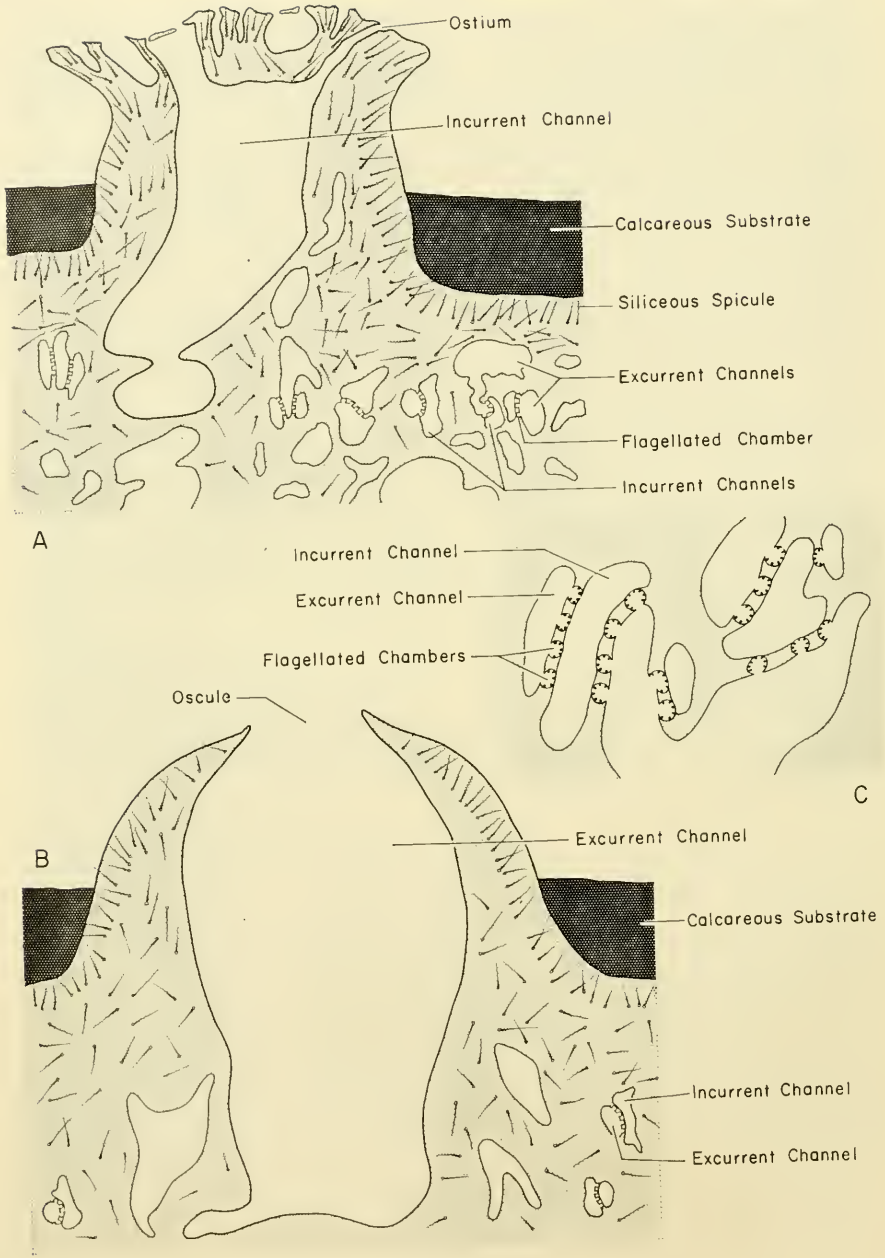

Ostium
Incurrent Channel
Calcareous Substrate
Siliceous Spicule
Excurrent Channels
Flagellated Chamber
Incurrent Channels
A
Incurrent Channel
Excurrent Channel
Flagellated Chambers
C
Oscule
Excurrent Channel
B
Calcareous Substrate
Incurrent Channel
Excurrent Channel

FIGURE 8

Morphology and Physiology of Clionid Sponges

Sponges of the family Clionidae live in cavities which they excavate in a variety of calcareous materials. Living or dead corals (Figs. 3 to 5) and mollusks, calcareous worm tubes, calcareous algae, and limestone are inhabited by the approximately 100 described species of this family. The genus *Cliona* with about 65 species is the best known of the 13 genera and will serve as the example for the remainder of this discussion.

In their basic structural and functional features sponges of the genus *Cliona* are essentially similar to other members of the Class Demospongiae, Phylum Porifera. Water currents are propelled through the sponge body in a system of canals by means of the beating of flagella borne individually on collar cells. Each flagellum is surrounded by a collar of cytoplasmic tentacles which help to direct the excurrent flow of water and also assist in the capture of food particles. Water enters the sponge by way of small pores or ostia and leaves by way of larger openings or oscules. The canals are separated into two discrete systems, an incurrent system and an excurrent system, and these communicate solely by way of flagellated chambers, hemispherical or thimble-shaped (Fig. 8C). Each chamber is composed of numerous collar cells, the flagella of which face the lumen and create a current toward the larger excurrent opening of the chamber. The water which is drawn in through the external pores of the sponge brings oxygen and food particles to the cells. Unicellular algae, bacteria, and possibly detritus make up the food of clionids as well as of most other sponges. The boring sponges, therefore, do not obtain nourishment from the soft parts of the animals whose shells or skeletons they attack. In some species the sponge may overgrow its calcareous substratum or destroy it completely and live subsequently as a non-excavating, free, massive individual (Fig. 2).

Fig. 8. Diagrammatic cross sections through boring stage of *Cliona celata* Grant. A, section through incurrent papilla (× 10). B, section through excurrent papilla (× 10). C, section through interior of sponge, showing relationship of flagellated chambers with incurrent and excurrent channels (× 96). (A, C modified from Vosmaer, 1933–1935.)

The clionids secrete a skeleton of siliceous spicules of three types: tylostyles, oxeas, and spirasters (Fig. 9). Tylostyles are present in all species; to these may be added oxeas or spirasters or both. The size and shape of the spicules as well as the configurations of the

Fig. 9. Siliceous spicules of *Cliona vastifica* Hancock. A, tylostyles; B, smooth or spined oxeas; C, spirasters. (From Hartman, 1958.)

excavations provide the most readily used characters for classifying these sponges.

In *Cliona* most of the cells of the sponge lie in galleries excavated within calcareous matter. But the incurrent pores and excurrent oscules must maintain contact with the external medium. Typically these openings are localized on papillae which protrude from holes excavated to the surface of the calcareous matter (Fig. 1). Numer-

ous pores open into each incurrent papilla, and a single oscule terminates each excurrent papilla (Fig. 8). The presence of the openings for papillae at the surface of the substrate provides the only indication of the presence of boring sponges in calcareous materials under natural conditions.

The Mechanism of Boring by Clionid Sponges

The unique quality of clionid sponges is their capacity to excavate calcareous material. The mechanism by which they accomplish this feat has been the subject of considerable investigation and much discussion on the part of zoologists since Grant (1826) described the first clionid, *Cliona celata,* living in oyster shells. The best observations of the boring mechanism are those of Nassonow (1883), who studied *Cliona stationis* (= *C. vastifica*) living in oysters in the Black Sea. He collected the sponge larvae in an aquarium and allowed them to settle on thin transparent flakes of oyster shell. Upon settling, the larvae flattened out and immediately began to bore into the calcareous substratum. The first evidence of this was the appearance of a group of etched lines outlining elliptical areas on the surface of the shell (Fig. 6). Nassonow said he could see cellular processes burrow into the shell following the course of the etched lines on the surface. Eventually small chips of shell were freed and voided to the exterior of the sponge. By the end of the first day of their existence as metamorphosed sponges, each of Nassonow's *Clionas* had excavated from 11 to 15 such particles from the shell fragment on which it had settled. These observations were confirmed by Warburton (1958), who planted fragments of *Cliona celata* on transparent calcite crystals and also allowed larvae of this sponge to metamorphose on the same substrate. He, too, noted the appearance of etched lines on the calcite underlying the sponge. The lines surrounded more or less elliptical areas, 35 to 45 microns in greater diameter, from which calcite fragments were eventually removed. Each fragment was bounded by several curved faces, convex or concave, meeting in sharp edges. Warburton also noted that if sponge fragments which had been allowed to attach to coverglasses were removed gently, the cells left behind "showed a re-

markable network of thread-like interconnections and pseudopodia, often 50 μ or more long. These surrounded areas reminiscent in shape of the calcite particles excavated by the sponges, or of the lines etched on calcite crystals."

Warburton dissolved away the calcareous components of an oyster shell riddled by *Cliona* and thus freed sheets of conchiolin, a proteinaceous substance lying between successive layers of calcium carbonate in the shell. He confirmed the observations of Topsent (1888) that the sponge is able to penetrate this substance and does so by removing particles comparable in size and appearance to the particles of calcite removed. Nassonov (1924) had also made this observation; both he and Warburton noted, however, that the sponge bored through conchiolin less readily than through $CaCO_3$. In oyster shells the sponges excavate extensive galleries through the successive horizontal layers of $CaCO_3$, but penetrate the intercalated sheets of conchiolin only at rare intervals.

Clionids thus excavate their galleries in calcareous materials by a relentless removal of small chips of $CaCO_3$. Not only are piles of such chips to be seen at the base of each oscular opening of the living sponge, but also the burrows themselves give evidence of the removal of numerous pieces of $CaCO_3$ by the shagreenlike appearance of their surfaces (Fig. 7).

How are the chips removed? Both mechanical and chemical explanations have been put forward, but there is no unequivocal evidence for either as yet.

Hancock (1849) supposed that the contractile powers of the cells caused certain siliceous bodies, about 42 microns across and embedded in the surface of the sponge, to grind away the calcareous matter. Later he (1867) recognized that the "siliceous bodies" were in reality fragments of conchiolin being excavated from the shell by the sponge. He then suggested that smaller angulated siliceous bodies, about 4 microns across and also embedded in the sponge surface, or the spicules themselves, must effect the excavation of the calcareous material. Hancock's theory (also maintained by Fischer, 1868) has now been abandoned completely, since Nassonow (1883; confirmed by Old, 1942, and Warburton, 1958) ob-

served the removal of calcareous chips by recently settled sponge larvae before spicules had developed.

Topsent (1888) believed that a purely mechanical action brought about by the contractility of the sponge cells was sufficient to explain the destructive activities of clionids. He argued that dissolution of the calcareous matter by an acid cannot be involved for two reasons: (1) conchiolin is removed in the form of small chips similar in size and shape to the chips removed from calcareous matter; and (2) the sponge cells in contact with the substrate differ in size from the calcareous chips removed, although he believed that the surface area of the chips should follow the outlines of the acid-secreting cells. In answer to Topsent's first point Cotte (1902), Nassonov (1924), and Vosmaer (1933–1935) have suggested that an enzyme may be secreted which attacks the organic components of mollusk shells. The observations of Nassonow (1883) on post-larval clionids indicated that protoplasmic processes of the sponge cells are the site of the boring activities of the sponge; thus the size of the cells need not correspond to that of the chips. Nassonow also noted certain elongate protoplasmic strands extending from adult clionids into the calcareous substrate, and thought that these were responsible for the extension of the excavations made by the sponge. Topsent (1887) pointed out that these latter protoplasmic prolongations are in reality boring algae or fungi and are quite independent of the sponge and its activities. He stated that these organisms are of common occurrence in dead bivalve shells whether they harbor boring sponges or not. It was remarked by Cotte (1902), however, that, although Nassonow was in error in his interpretation of the elongate strands seen extending from adult sponges, he was correct in his observations of cell processes extending into the calcareous substrate in his postlarval preparations. In a study of decalcified sections of *Cliona vastifica* boring in a mollusk shell, Cotte saw pseudopodial expansions of mesenchymal cells extending beyond spaces between the outer epidermal cells of the sponge where these are in contact with the substrate. He noted that these mesenchymal cells are identical with the spherule-containing cells described by Topsent (1900), stating that he could see granules in

the pseudopodia like those in the cell body. Cotte agreed with Nassonow in ascribing to these cell processes the role of dissolving away the calcareous substrate in such a way as to remove small chips of $CaCO_3$. As noted above, Warburton (1958) also observed a network of pseudopodia extending from clionid cells in contact with the substrate.

Letellier (1894) supposed that the contractility of the cells adhering to the substrate brings about sufficient pressure to break away small particles of $CaCO_3$. He cemented small threads or rods of rubber and gutta-percha to oyster shells and found that by twisting these in various directions he could eventually break away chips of shell with shapes similar to those removed by clionids.

Nassonow (1883), Cotte (1902), Vosmaer (1933–1935), and Warburton (1958) have all proposed that the chips are removed by a localized dissolution of calcareous material at the point of contact between the cytoplasmic extensions of the cells and the substrate. As yet no investigator has demonstrated a lower pH at these points of contact, and there is no clear-cut evidence showing an increase in dissolved calcium in media in which clionids have been cultured (Old, 1942; Warburton, 1958). Vosmaer (1933–1935) has suggested that carbonic acid produced by the sponge is the substance most likely to be responsible for the dissolution of the calcareous substrate.

In summary, it may be said that attempts to explain the boring activities of clionids in terms of mechanical abrasion by spicules have been ruled out by the observations of Nassonow (1883), Old (1942), and Warburton (1958) on postlarval sponges lacking spicules. That the adhesive and contractile properties of the sponge cells alone can bring about the excavations seems unlikely as well. Cotte (1902) has pointed out that in old shells weakened by a network of algal borings, if forces of traction were exercised by clionid cells, the chips broken away would be irregular in shape. Instead, in such instances, the lunules of $CaCO_3$ removed from the substrate are identical with those which occur when the sponge bores into calcite. Warburton (1958) has noted that, if mechanical action of the type described is involved, it would be expected that the chips would be severed along planes parallel to the cleavage faces of

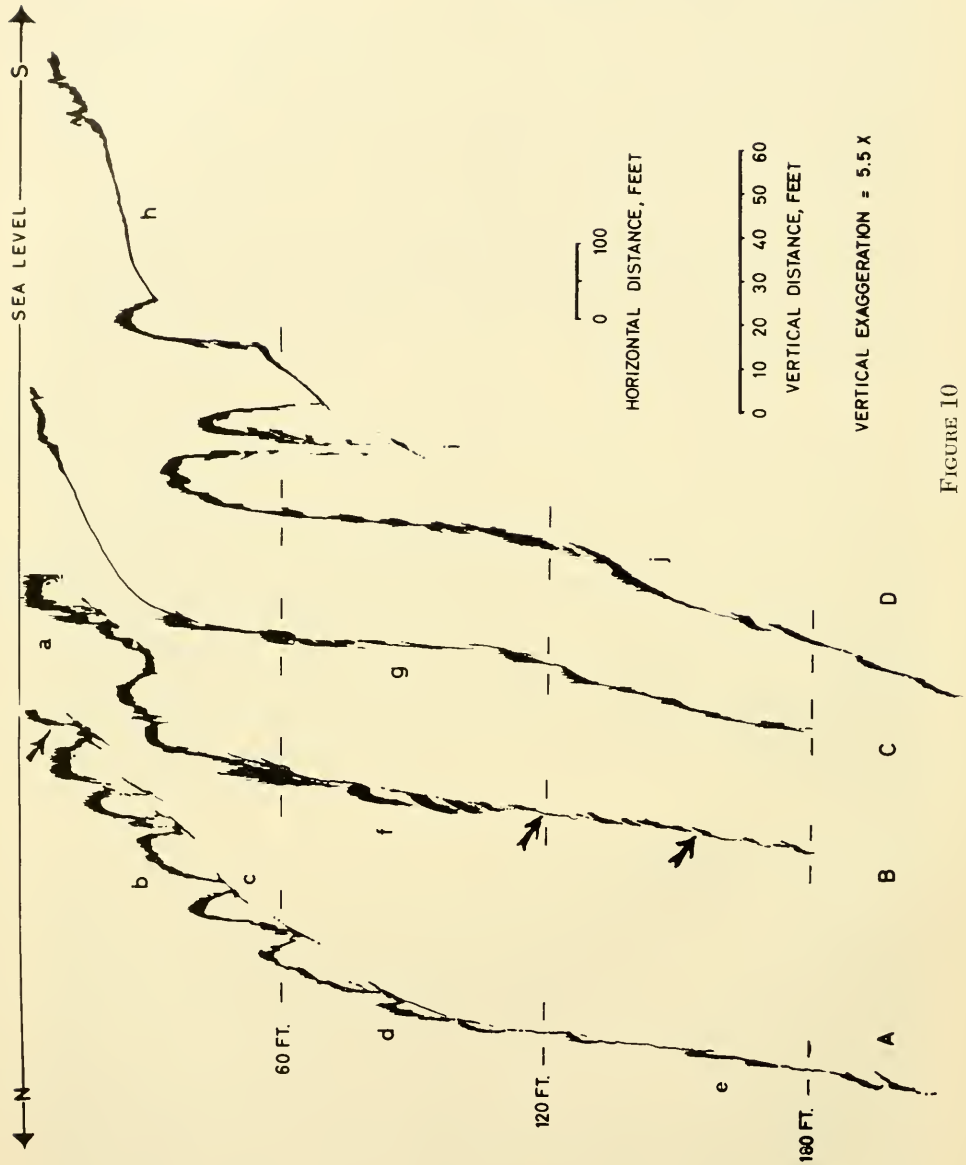
crystalline calcite when this serves as a substrate for the sponge. Instead, the chips are bounded by curved faces and have the same shape whether the sponge bores into mollusk shells, corals, limestone, or Iceland spar. It is highly probable that the removal of chips from the substrate by clionids results from localized chemical dissolution of the calcareous matter where cell processes come in contact with it. The nature of the chemical process involved has not yet been demonstrated. It is possible, as Nassonow (1883) and Vosmaer (1933–1935) have noted, that the contractility of the sponge cells plays an auxiliary role in the removal of the chips along with a chemical process.

Structure and Ecology of Coral Reefs in Jamaica

The field studies described here were made by SCUBA-equipped divers on the north coast of Jamaica, between Rio Nuevo and Duncans. The reefs of this area are heavily buttressed (Goreau, 1959, 1961; Zans, 1958) and are built up on the edge of a near-vertical offshore slope which falls to oceanic depths within a kilometer from the shore. The submarine topography of these reefs and their associated structures is shown on the fathogram profiles in Fig. 10. The principal characteristics of the major biotic zones are summarized below.

The Reef Crest, 0 to 20 Meters

The reef crest includes the shallow rampart, the surf zone, and the buttress zone to a depth of about 20 meters on the seaward side. The environment is of the high-energy, high-light-intensity type, as it lies in the shallow, well illuminated region above the wave base. The dominant organisms are the reef corals, which reach very large sizes (see Fig. 11) and build a massive wave-resistant framework raised above the surrounding sea bottom (Lowenstam, 1950). Coral and algal $CaCO_3$ production is so high in this region that the overflow governs the pattern of carbonate sedimentation over a widespread area outside the reef. The accessory fauna of sponges, Gorgonacea, and Bryozoa is reduced; boring organisms are common but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D
0 100
HORIZONTAL DISTANCE, FEET
0 10 20 30 40 50 60
VERTICAL DISTANCE, FEET
VERTICAL EXAGGERATION = 5.5 X

FIGURE 10

their erosional effects are compensated by rapid overgrowth of corals and cementing coralline algae.

The Fore-Reef, 15 to 30 Meters

This region interdigitates with the base of the reef buttress but is distinguished from it by having little primary framework. The depth is great enough so that wave surge is much reduced, yet there is sufficient turbulence to prevent accumulation of fine reef silt; it is a medium-energy environment with the ambient light intensity averaging about 25 per cent of the surface value. The coral fauna is still extremely varied as regards species number, but colony size and population density are decreased, and much of the available space is occupied by sand-producing calcareous algae, sponges, and large Gorgonacea.

The Deep Fore-Reef Slope, 25 to 70 Meters

This zone is the seaward extension of the fore-reef and is the transitional region between the true shallow-water coral reef province and the deep-water biotopes of the oceanic slope. An important physical property of this habitat is its precipitous topography: the gradient is seldom less than 45° and often more than 70°. The main drop-off faces north in this part of Jamaica, thus the area is badly illuminated and the bottom light intensity at the 50-meter level is estimated to be about 5 per cent of the surface value. There is in-

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Fig. 10. Topographic profiles from fathometer surveys on Jamaican reef systems where extensive erosion of coral by boring sponges was observed. Sections A and B are profiles of reefs off Mangrove Point and Maria Buena Bay near Duncans; sections C and D were taken off Eaton Hall and Cardiff Hall at Runaway Bay. The lower-case letters indicate major reef features referred to in the text: *a*, shallow coral framework of the reef crest; *b*, reef buttress composed of spurs and chutes, *c*; *d*, fore-reef; *e*, fore-reef slope with scattered large coral aggregates; *f*, slump due to collapse of the basal reef structure onto the outer slope; *g*, near-vertical submarine cliff with rich coral and sponge biota; *h*, lagoon-type sand bottom sloping away from the shallow inshore reef at right; *i*, gap in reef due to drowned river valley of Pleistocene age; *j*, detrital cone of shallow-water reef talus transported onto forward slope through gap at *i*. The arrows on profiles A and B indicate the approximate location of the underwater scenes shown in Figs. 11, 12, and 13. To find the distances in meters, multiply the scale distances by 0.3045.

FIG. 11. Underwater photograph of a typical shallow-water coral association in the reef buttress at a depth of about 4 meters, off Mangrove Point, Jamaica. These corals are growing in the turbulent zone and have built a wave-resistant primary framework. Various types of branching, foliose, and massive corals are shown. Most of these are large, heavy, and extremely well cemented to the substrate. The canyon in the middle background is about 7 meters deep and contains large branches of dead elkhorn coral broken off during storms.

tensive sedimentation due to transport of mixed reef detritus from the shallow zones above. In localities where the slope is less than the angle of repose, immense areas of the bottom are covered with thick layers of fine muddy sand which is devoid of corals. Where the angle of repose is exceeded, mass flow of sediments downslope prevents accumulation and exposes outcrops of the underlying rubble and rock strata. The latter provide a firm base for sessile organisms including algae, Foraminifera, sponges, Gorgonacea, Antipatharia, Bryozoa, bivalve mollusks, tube worms, etc. Weak detrital cementation is caused by encrusting Foraminifera and by lithothamnioid algae.

Reef coral communities occur on the outer slope wherever bottom conditions are favorable, but as the depth increases below 30 meters the coral growth becomes patchier, with progressive reduction in species number, colony size, and population density. Some typical fore-reef slope communities are shown in Figs. 12 and 13. Primary frame building does not take place here because of the lack of large, massive coralla, the fast rate of erosion by boring organisms, and the paucity of encrusting calcareous algae, which are such important frame cementers in shallow water.

Colony Form and the Attachment of Reef Corals in Shallow and Deep Water

Before describing the effects of boring sponge erosion on deep coral populations, it is necessary to point out that the corals of the fore-reef slope differ strongly from their shallow-water relatives in colony shape, attachment to the substrate, and encrusting biota.

Colony Shape

The most striking property of corals on the fore-reef slope is their flat shape; virtually absent are the massive hemispheroidal forms so typical of the same species on the shallow reefs. The change from a three- to a two-dimensional growth plan is correlated with reduced environmental light intensity, and has been observed only in corals with zooxanthellae (Goreau, 1963), for example *Siderastrea, Diploria, Stephanocoenia, Mussa, Mycetophyllia, Dicho-*

FIG. 12. Reef coral association at a depth of about 40 meters on the fore-reef slope. The position of this steep submarine declivity is shown by the upper arrow on profile B in Fig. 10. The flat, platelike corals are *Agaricia undata*, here growing on a large heap of unconsolidated talus. These corals are not building primary framework; indeed, the colonies were poorly attached to the bottom, and heavy destruction by boring sponges was in evidence. The tall objects in the middle are sponges, and the wiry growths on the overhang are Antipatharia.

FIG. 13. Climax community dominated by the deep-water reef coral *Agaricia undata*, at a depth of 50 meters, Maria Buena Bay, Jamaica. The coral population in the foreground actually consists of a close association of *A. undata* with *A. cucullata*, *A. fragilis*, and an undescribed species of *Agaricia*. This type of coral community occurs only on the open fore-reef slope in the presence of coarse reef talus. The outwardly solid appearance of the cabbage-like coral growth hides the extensive damage done to their bases by boring sponges. The feathery growths in the background are Gorgonacea, and several large sponges are visible below and to the left of the diver. The approximate location of this scene is indicated by the lower arrow on profile B in Fig. 10.

coenia, and *Porites,* all of which normally build large, massive rounded heads in well illuminated environments. Foliose and branching genera, such as *Agaricia, Acropora,* and *Madracis,* become thinner and more spindly but do not undergo marked changes in geometry. The outstanding consequence of flattened growth is that such colonies are much lighter in proportion to their surface area than round forms of the same radius (Goreau, 1963).

Attachment of Corals to the Substrate

The base of shallow-water corals is usually skirted by a layer of living polyps, so that the attachment grows in proportion to the bulk of the corallum: the larger the corals, the better cemented they are and the more difficult it is to separate them from their substrate. In deep water, however, only the upper surfaces of the flattened colonies are alive, and the holdfast is not covered by a layer of polyps; the growth of these colonies therefore is not accompanied by a proportional increase in thickness and strength of the base, so that large individuals are often precariously perched on a thin neck which is easily broken.

Encrusting Biota

The dead, exposed undersurfaces of deep-water corals also form ideal sites for sponges, hydroids, algae, Bryozoa, Gorgonacea, Antipatharia, Foraminifera, worms, and mollusks. The majority of these organisms merely use the coral as a foothold, but they often reach such large sizes that the supporting colonies are overwhelmed. Indeed, the thin attachment of the coral can become so overloaded that it will break when the mass becomes too heavy. Figures 14 and 15 show the characteristic narrow base of flattened, sponge-encrusted corals growing on the outer fore-reef slope.

The Effect of Boring Sponges on the Corals of the Fore-Reef Slope

Damage by boring sponges is widespread in all reef zones; most corals show evidence of penetration at sites where the skeleton is not protected by living polyps. Deep-water corals, with their thin-

FIG. 14. Lateral view of an extremely flattened colony of *Agaricia* growing at a depth of about 30 meters near Runaway Bay, Jamaica. This thin, sheetlike coral was attached to the rock spur in the middle by a slender neck so riddled with borers that gentle pressure from one hand was sufficient to break it off. Sponges can be seen hanging down from the underside of the coral. The large vase-shaped sponge is about 30 cm tall.

FIG. 15. Side view of greatly flattened specimens of *Porites astreoides* and *Montastrea annularis* at a depth of about 30 meters on the steep outer slope. These species are abundant throughout the reef, but in shallow water their colonies are larger, heavier, and hemispheroidal in shape. The corals shown here were much damaged by boring organisms and also carried a variety of large sponges attached to dead parts of the colonies. The tube sponges hanging from the underside of the lower coral were almost 1 meter in length.

ner skeletons, slower growth rates, and large dead areas around the base, are more riddled with sponge burrows than are shallow-water colonies of the same size. The infestation starts in the older parts of the exposed skeleton around the base and may spread to form a network of cavities ramifying throughout the colony.

There is no conclusive evidence that excavation of the nonliving interior of corals by boring sponges has any injurious effect on the polyps per se. The survival of the colony as a whole is not seriously jeopardized until the attachment of the coral is weakened to the breaking point. The fate of corals after they are dislodged depends on the species and on the environment: on level bottom the colonies may remain in place but will suffer injury if they are tilted on their sides or turned over; in steeply sloping areas the loose colonies slide or roll into deep water and are killed by burial in the sediments. Corals encumbered by heavy growths of attached organisms may be at a considerable disadvantage because of the extra load on the already weakened base, but our observations also show that large encrusting sponges often support and hold such corals in place long after their original holdfasts have been eroded away by boring organisms.

The loosening of corals through destruction of their basal parts is so marked at depths below 50 meters that large living colonies can be collected easily by hand without the use of tools. Such individuals are only able to maintain their position on steep slopes by virtue of their light weight, and because the extreme flattening provides a broad area of contact that becomes snagged on surface irregularities of the substrate. The flattening also prevents the corals from turning over. Under similar conditions, the heavier rounded types of coral are unable to remain in place, since they roll downslope into deep water as soon as they come adrift. It is therefore clear that the flattening response in reef corals has considerable adaptive value in environments where heavy loss to the coral population is caused by the combined effects of boring sponge activity and precipitous bottom topography.

The Role of the Boring Sponges in the Erosional Remodeling of Coral Reefs

On the steep outer slopes where normal wave action is negligible, the displacement and drainage of loose skeletal detritus out of the reef communities occurs mostly under the impetus of gravity. Our observations on sediment distribution in these habitats, to be described elsewhere in greater detail, show that the mass transport of material eroded from reefs takes place in at least four different ways, all influenced in varying degree by the activities of the boring sponges.

Talus Formation

Reef associations continually produce large quantities of skeletal debris ranging from big corals to mud-sized aragonite needles. This material gravitates downward and distributes itself on the outer slope according to size, the heavy coralla remaining where they fall to build up a steeply inclined scree zone flanking the lower edge of the living coral belt, the lighter fine sediments drifting farther down to accumulate in thick beds of muddy sand spread over the lower reaches of the fore-reef slope. Talus fallout occurs at all times, even in calm weather. Corals have been seen to tumble down, apparently coming adrift through spontaneous fracture of the base. Such freshly fallen corals as we have inspected, however, were invariably riddled with boring sponges, and it is probable that this was the main cause of their accidental detachment. The forces initiating coral fallout need not be large; indeed, the weak turbulence produced by the swimming movements of the divers is sometimes enough to loosen the colonies and start them sliding down.

Coral Slides

In contrast to simple fallout, which involves both fine and coarse detritus, large quantities of boulder-sized talus are transported by underwater landslides, especially in areas where rich coral associations have become established on high submarine cliffs. Such slides leave characteristic traces; the cliff face shows localized areas of denudation (Fig. 16) and the fore-reef slope below is covered by

conical detritus heaps where the slide masses have come to rest. We have no direct evidence on how such movements originate; it is probable that they are set off by storms when the surge depth is greater than normal. Because deep-water coral associations do not build wave-resistant reefs, the combined effects of unusually heavy wave action and structural weakening by boring sponges are likely to have catastrophic consequences: under such conditions, corals cascading down in large numbers may spread destruction rapidly by means of successive collisions that culminate in major slides and avalanches.

Subsidence and Slump

This form of reef erosion differs from slides in that it is a slow subsidence of intact reef masses over vertical distances as great as 20 meters. The unstable region is usually delimited by large cracks along planes of slippage. Differential movement occurs by repeated block fracture, resulting in the formation of slump terraces, some of which are shown on the fathometer profile B in Fig. 10. Reef slump in Jamaica is most common at depths below 20 meters where vigorously growing fore-reef communities are perched on the edge of a drop-off, and large, poorly consolidated coral promontories are built out over the edge with their bases resting on unstable fine sediments. At such depths cementation by algae is slow, whereas boring sponge activity is intense; hence the coral frame is exceedingly fragile. We have no information as to the nature of the forces that trigger off subsidence: on the one hand, it may be due simply to the progressive accumulation of an unstable mass of coral; on the other hand, earthquake shocks may cause initial slip along planes of weakness.

Sediment Flow

Besides coarse talus, reef associations also produce vast quantities of fine skeletal debris that is winnowed out and deposited in thick sand beds outside the community proper. In the turbulent shallow zones the sediment is coarse and well sorted, but in calm deep water there is less sorting and a large proportion of mud is present. Drainage of the fine calcareous residue from reefs is essential to the well-

FIGURE 16

being of the sessile communities: in shallow water, wave turbulence is the prime mover; in deep water, it is mainly the force of gravity that induces flow on sloping surfaces. The mobility of the sediments is enhanced by the lubricating and fluidizing effect of the mud fraction, the origin of which is still very poorly understood.

Preliminary studies near Runaway Bay have shown that the fore-reef slope sediments immediately below coral communities contain a much higher proportion of calcareous mud than those farther away, and that the accumulation of mud is greatest where the growth of corals, algae, and sponges in the overlying reefs is best. Although there appears to be a general relationship between mud formation and biological activity in coral reefs, it is not yet known what specific mechanisms are involved. It is probable, however, that the ability of boring sponges to reduce massive skeletal carbonates to very small particles is of major importance in the production of fine sediments in the reef habitat.

So far, the greatest emphasis has been on the destructive aspects of boring sponge activity, but the same processes which promote the breakdown of reefs also make possible the establishment and growth of large coral communities on the deep slope. The coarse, sponge-riddled detritus transported onto the fore-reef slope provides an ideal foundation for highly specialized sessile populations that could not under normal circumstances colonize this habitat. The dominant coral of the deep talus belt is *Agaricia undata*, a species restricted almost wholly to localities where avalanche detritus has been deposited over the soft and barren sediments of the open fore-reef slope at depths between 35 and 75 meters. Here, *A. undata* aggregates with other agariciid corals such as *A. cucullata*, *A. fragilis*, and an undescribed species to build up large compound

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FIG. 16. A submarine cliff recently stripped of corals by an avalanche. The area on the left has been recolonized by sponges, algae, and Antipatharia, but corals are still lacking though they are abundant on adjacent parts of the cliff. Below the diver is the top of a slide mass of coral detritus. Unlike the older talus heaps shown in Figs. 12 and 13, this detrital mass has not yet been populated by corals. The treelike object in the foreground is a large antipatharian on which some sponges are also growing. This photograph was taken at a depth of about 35 meters near Cardiff Hall, Jamaica.

colonies (Fig. 13) of a type never seen in any other part of the coral reef biotope. The deposition of coarse reef material on the fore-reef slope appears to be an essential requirement for the subsequent establishment of these deep-water *Agaricia* communities. In a more general sense it seems that detrital slides and avalanches must constitute an important mechanism whereby conditions favorable for the development of corals are extended into environments normally inaccessible to many sessile organisms because of the lack of a stable, well drained substrate.

It is remarkable that on the fore-reef slope there is no formation of a primary framework (Goreau, 1963) despite the productive reef-building fauna and flora. The immense coral-encrusted detritus heaps described above are not in any sense to be thought of as primary coral framework comparable to, say, the buttresses of the reef crest, since they are predominantly not the result of *in situ* coral deposition, and there is almost no structural consolidation by cementing organisms. The lack of framework growth on the outer slope is due to (1) slow rate of calcification with flattening of corals, (2) paucity of frame-cementing biota, (3) intensive erosion. Of all these factors, there is no doubt that the erosional processes initiated, and facilitated, by the boring sponges are most important. By weakening their attachment to the substrate, the boring sponges preferentially accelerate the loss of the larger and more massive corals which are also the major potential frame builders.

Not enough information is yet available to determine whether the ecological triad described above, i.e., steep topography plus reduced frame-building capacity plus boring sponge activity, occurs elsewhere: the sharp offshore precipices bordering coral reefs in the Red Sea (Nesteroff, 1955) and the Pacific Ocean (Emery *et al.*, 1954) suggest that the processes we have observed in Jamaica may be much more widespread than is now believed.

Summary

1. Intensive invasion of corals by boring sponges has been observed in reef communities at depths below 30 meters on the fore-reef slope in Jamaica. In this habitat the consequences of large-scale

boring activity are more obvious than in shallow water because biological calcification is slow and wave surge is negligible.

2. Boring sponges burrow into any substrate composed of aragonite or calcite, i.e. shells, corals, and limestone. In corals, the sponges erode only the nonliving skeleton; the living polyps are unaffected. The older parts of the colonies are most damaged, especially the holdfast, which is thin and fragile in deep-water reef corals. The larger colonies often break off under their own weight and fall into deep water, where they are killed by burial in soft sediments.

3. Most deep-water reef corals have a thin and flattened colony shape. On account of their light weight and broad area of contact with the substrate, such platelike colonies often remain in place after their holdfasts have been eroded away, whereas heavy rounded coral heads will roll downslope and be lost. It is suggested that the flattening response, believed to be an effect of low light intensity on skeletogenesis, has considerable adaptive significance for reef corals growing in deep water under conditions where heavy destruction by boring sponges and precipitous bottom topography occur together.

4. The widespread boring out of corals and the concomitant softening of the limestone substrate weaken the entire reef and make it more susceptible to the destructive action of storm waves, earthquakes, and so on. Mass transport of loosened corals and coarse detritus from the upper reef to the deep fore-reef slope is due to (*a*) talus fall, (*b*) slides and avalanches, (*c*) reef subsidence and slump.

5. The deep fore-reef slope is normally covered by thick layers of bare muddy sand devoid of corals. However, when large amounts of coarse reef talus are deposited over the soft sediments of the outer slope, a stable, well drained substrate is provided on which dense coral populations subsequently become established. The deposition of reef block talus in deep water thus results in an extension of environmental conditions advantageous for the growth of coral communities into regions where they cannot otherwise exist.

6. It is suggested that the very fine calcareous flakes ejected by active boring sponges contribute to the large volumes of mud pro-

duced in reef communities. This fraction may also have important lubricating and fluidizing properties which speed the drainage of skeletal sediments from the reef.

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3

Demineralization Mechanism of Boring Gastropods

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A WIDE range of species in at least eight invertebrate phyla and three major plant groups have the capacity to excavate the calcareous exoskeletons of other organisms. The nature of the mechanism by means of which this is accomplished is little understood. It is not known whether the mechanism is fundamentally similar in predatory boring gastropods and octopods which penetrate bivalves for food; in bivalves which excavate shelters in the valves of mollusks; in sponges, flatworms, bryozoans, phoronids, polychaetes, and barnacles which excavate calcareous substrates for nutrients and/or protection; in shell-boring fungi, algae, and bacteria; and in microorganisms associated with human dental caries. Among plants and lower invertebrates, penetration of calcareous substrates is probably achieved by chemical means alone; in higher invertebrates, by mechanical or chemical means or a combination of these. Various hypotheses suggest that release of CO_2, acids, enzymes, or sequestering agents may bring about dissolution of shell. In higher inverte-

brates mechanical devices such as valve edges in pelecypods, radulae in gastropods, and bristles in polychaete and sipunculid worms and in barnacles may be employed alone in penetration of relatively soft materials; but perforation of hard exoskeletons probably requires chemomechanical means (Carriker, 1961).

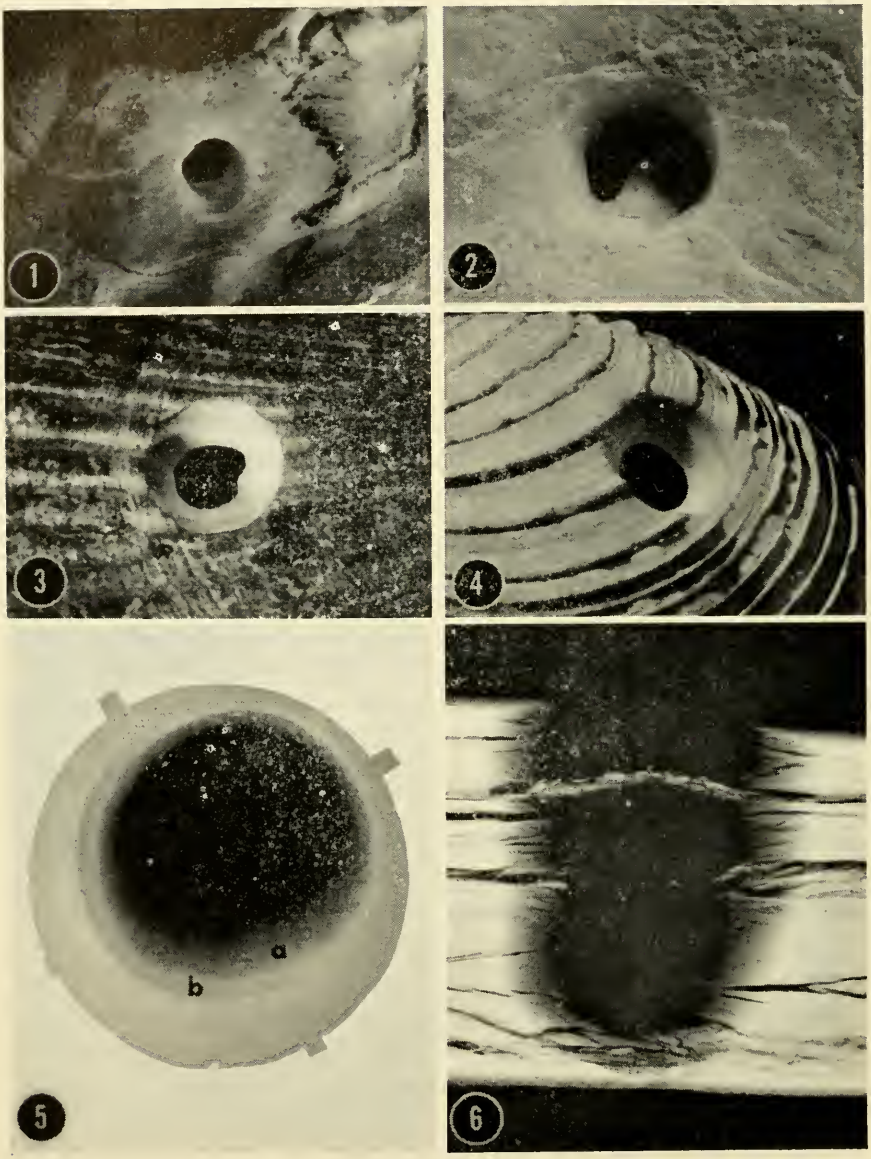
Since 1941 the senior author has been studying the biology of the demineralization-boring mechanism of drilling gastropods in an attempt to gain a better understanding of the mechanism in these snails, and to provide a basis for a wider comparative study of similar mechanisms in other mollusks and in other invertebrates (Carriker, 1943). Recent species of gastropods which bore are all predatory, feeding on the bodies of animals enclosed within shells, and are known to exist mainly in the Families Muricidae (including Thaididae) and Naticidae, Subclass Prosobranchia; continued exploration may well disclose them in other gastropod families.

As currently understood, drilling in muricid and naticid snails consists of a chemical phase in which an accessory boring organ, ABO (demineralization gland), secretes a neutral substance which acts on the shell at the site of penetration, and a mechanical phase in which the radula rasps off the weakened shell as minute flakes which are swallowed (Carriker, 1961). Descriptions of various aspects of the anatomy, histology, and function of the organs involved in boring in muricids are given by Carriker (1943, 1955, 1957, 1959, 1961), Fretter (1941, 1946), and Graham (1941); in naticids by Ankel (1937, 1938), Boettger (1930), Fischer (1922), Giglioli (1952), Gunter (1936), Hirsch (1915), Schiemenz (1891), Turner (1953), and Ziegelmeier (1954); and in both families in a review in an impressive new volume by Fretter and Graham (1962). Many aspects of this biology are still grossly incomplete.

In muricids the ABO, withdrawn within a sac in the mid-anterior part of the sole of the foot, is everted only in operation; in naticids it lies under the distal tip of the proboscis. The ABO takes the form of a papilla, mushroom-shaped in muricids and pad-shaped in naticids, and has a distal cap of a very tall distinctive secretory epithelium, strikingly different from other epithelia in the snail body. The diameter of the ABO slightly exceeds that of the tip of the proboscis, and approximates the diameter of the bore hole made by it.

The boring function may be reviewed briefly as follows. Drilling gastropods readily locate prey in their immediate surroundings, appearing to orient to external metabolites emitted by them (Blake, 1960); the manner of identification of the metabolite signal, and of the prey after the snail has mounted it, is not known. Once contact has been made with prey and a drilling site on it has been selected, the snail rasps this free of incrustations, periostracum, and loose shell material with the radula; then it brings the ABO immediately over the drilling site, and extends the organ into the shallow concavity. The foot during this time adheres strongly to the prey, and completely hides and protects the ABO. While the ABO is everted in the concavity, there is no noticeable external movement of the snail, and its pedal surface, firmly applied over a copious film of mucus to the intact surrounding shell, appears to effect a watertight connection. After an interval of a few minutes in Naticidae, and as long as an hour in Muricidae, the ABO is gradually withdrawn, and the snail explores the surface of the excavation and close surroundings with the proboscis tip and propodium. Rasping within the region affected by the ABO is then resumed and continues for a few minutes; the proboscis is held snugly in a groove formed by infolding of the propodial folds. Radular teeth are rather ineffectual in removing untreated shell, as demonstrated by the fact that flakes of shell are not removed at the close of the rasping period. Alternation of periods of rasping and chemical action continues until a perforation is completed, and the snail then feeds on the flesh of the prey through the hole it has drilled. By removal of the ABO and the radula in different individual muricids, Carriker (1959) demonstrated that penetration involves obligatory alternate use of the radula and of the demineralization organ. When functioning, the ABO is swelled by hemostatic pressure and is extended to the deepest extremities of the hole, maintaining the distal secretory epithelium in close contact with the shell substance at the bottom of the hole.

Perforations made by boring gastropods are characteristically smooth and circular, penetrate the shell at right angles to the surface, and may decrease noticeably in diameter at the inner end. The outer parts of naticid borings are broadly beveled, converging



FIGURES 1 TO 6

toward the interior of the shell (Figs. 3 and 4), and incomplete holes have a central boss. On the other hand, muricid bore holes are not beveled or only slightly so, are straight-walled (Figs. 1 and 2), lack the central boss, and often contain shallow shelves of organic matrix. Circularity of the holes is obtained by the radula as it is rotated on the long axis of the proboscis by buccal muscles. Size of holes varies with species and size of individuals; and form, with species and with ornamentation of prey shell (Bucher, 1938; Carriker and Boone, 1960; Fischer, 1922; Ziegelmeier, 1954). Although the naticid and muricid bore holes studied to date appear diagnostically distinct, the holes of many other species in both families will have to be examined before this observation can be confirmed. Rate of penetration of shell is approximately 0.25 mm per hour in Naticidae (Ziegelmeier, 1954); rates have not been reported for Muricidae.

Ankel (1937) and Carriker (1961) have excised the ABO from some 20 different species of living boring muricid and naticid gastropods, and these glands when applied to the smooth glossy inner surface of mollusk shell (either calcite or aragonite) produced etch-

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FIG. 1. Bore hole, 1.8 mm in outer diameter, drilled by *Eupleura caudata etterae* in shell of *Crassostrea virginica*. (× 5.)

FIG. 2. Bore hole, 4 mm in outer diameter, drilled by *Murex brevifrons* in shell of *C. virginica;* the incomplete perforation at bottom of hole is characteristic of this species. (× 5.)

FIG. 3. Bore hole, 3 mm in outer diameter, drilled by *Polinices duplicatus* in shell of *Modiolus demissus*. (× 6.)

FIG. 4. Bore hole, 3 mm in outer diameter, drilled by *P. duplicatus* in shell of *Mercenaria mercenaria*. (× 6.)

FIG. 5. Projection microradiograph of ground section 0.7 mm thick cut parallel to surface of shell of *C. virginica* with hole drilled by *Urosalpinx cinerea follyensis;* hole 1.4 mm in diameter, and midway through shell. Study of the section by stereographs indicates that the inner radiolucent area (*a*) is due to changes in the contour of the hole through the section, and that the outer radiolucent region (*b*) is a zone of partial demineralization surrounding the hole. Ti radiation; 25 kv, 30 μ aperture. (× 25.)

FIG. 6. Projection microradiograph of ground section cut at right angle to surface of shell of *C. virginica* with hole drilled by *U. c. follyensis;* section was cut to one side of the hole to demonstrate zones of dense conchiolin extending as ledges into the lumen of the hole; the peripheral demineralized zone illustrated in Fig. 5 is also shown in this section. Width of the portion of the hole shown here, 1 mm. Ti radiation; 20 kv, 45 μ aperture. (× 25.)

ings in a few minutes to several hours. These ranged from faint markings to definite shallow indentations in the shell, and they assumed the diameter and form of the distal disk of the organ. The secretion altered the gloss of the shell, leaving a conspicuously roughened surface.

The secretion of the ABO has not yet been characterized. Investigations by Ankel (1938), Fischer (1922), and Hirsch (1915) in which fluids from the organ and from incomplete perforations were tested with pH indicator papers, and fluids from homogenates of the organ were titrated with NaOH, indicated a neutral reaction.

The fact that acids have not yet been identified in the secretion of the ABO of either Muricidae or Naticidae, the suggestion of Hirsch (1915) that a calcase may be present in the secretion, and the experimental use of chelating agents for demineralization (Galtsoff, 1955; Grégoire, 1957) have prompted the hypotheses that in the chemical phase of boring the glandular epithelium of the ABO may secrete a conchiolinase which might act upon the matrix of shell, or a calcase and/or a chelating agent which might solubilize the mineral component, or both. (See Wilbur, 1960, for a discussion of the structure of mollusk shell.) Since boring also involves mechanical rasping, chemical agents need not completely destroy the shell preparatory to removal by radulae. Weakening of either the organic or the mineral components would permit removal of shell material by radular denticles.

Since 1960 we have been conducting a joint study of the effect of the ABO secretion of muricid and naticid snails on calcareous substrates in an effort to provide a preliminary characterization of this secretion and to contribute further information on the demineralization-boring mechanism. These investigations have included principally microradiography of sections of bore holes; physiological, optical, and electron microscopic study of the action of the excised ABOs and ABO homogenates on a variety of calcareous substrates; and a comparison of these effects with the action of several artificial agents on the same substrates. The present paper reports progress made in these studies. Comparative morphological, cytological, cytochemical, and ultrastructural investigations of the ABO itself are

now being carried on; and biochemical characterization of the ABO secretion is planned.

Materials and Methods

Boring Gastropods

The following species of snails were employed (scientific names of mollusks used in this paper are taken from the nomenclature of Abbott, 1954):

Family Muricidae. *Urosalpinx cinerea follyensis* and *Eupleura caudata etterae* (B. B. Baker, 1951) are large forms of the species found only along the embayments of the Eastern Shore of Maryland and Virginia. The height of *U. c. follyensis* ranged from 28 to 45 mm; that of *E. c. etterae*, from 28 to 40 mm. Both species were collected in Chincoteague Bay and in Wachapreague Bay in relatively shallow water, where they were plentiful. Most ABOs for experimental studies were excised from *U. c. follyensis*.

Murex fulvescens were collected by diving in water ranging in depth from 1 to 8.5 meters below mean low water on a rock jetty inside Shackleford Banks, North Carolina. The largest specimens ranged in height from 9.5 to 12 cm.

Murex brevifrons, a tropical species, were collected in the vicinity of Boqueron, Puerto Rico, where they were plentiful, and were shipped alive by air mail to us in North Carolina. The height of specimens ranged from 6.5 to 10.5 cm.

Snails were maintained in our laboratory in running or frequently changed recirculated sea water ranging in salinity from approximately 28 to 32 ‰. They adjusted easily to captivity, and fed voraciously on small to medium-sized eastern oysters, *Crassostrea virginica*.

Family Naticidae. *Polinices duplicatus* and *Sinum perspectivum* were collected on the expansive intertidal sand flats of Bird Shoal, Beaufort area, North Carolina, where the former species was abundant. The largest specimens of *P. duplicatus* used were 4.5 cm in

shell height; the length of the extended foot of the largest *S. perspectivum* employed was 7.5 cm. These snails were held in running sea water and given live hard clams, *Mercenaria mercenaria,* for food.

To obtain incomplete bore holes of species in both families we interrupted the snails in the act of penetrating live bivalve prey.

Calcareous Substrates

Selected areas of the smooth nacreous inner surface of the valves of the oyster *Crassostrea virginica,* chiefly $CaCO_3$ in the form of calcite (sometimes referred to as calciteostracum) (Wilbur, 1960), and the highly porcelaneous glossy inner surface of the univalve of the muricid snail *Murex fulvescens,* mainly $CaCO_3$ in the form of aragonite, were used principally as substrates in the study of etchings by ABOs. X-ray diffraction powder patterns were made from samples of the outer prismatic and inner nacreous layers of *C. virginica* and *M. fulvescens;* calcite was found in both layers of the former species, and aragonite in the latter. Etchings were also made on the valves of the clams *Dosinia discus* and *Spisula solidissima.*

For nonbiologically formed crystals of $CaCO_3$ with no organic matrix we used the flat, cleanly cleaved glossy surfaces of large pure mineral calcite and aragonite crystals. Polished surfaces of enamel and dentin of cross-sectioned human teeth provided a calcium phosphate substrate in the form of hydroxyapatite crystals.

Organic Substrates

Preparation of demineralized conchiolin of mollusk shell is performed without difficulty by suspending shell in a saturated solution of EDTA (disodium ethylenediamine tetraacetate), pH about 6, in a tall cylinder at room temperature. An oyster valve 2.5 cm in diameter may be decalcified in about 2 days, leaving a highly translucent firm gelatinous mass of conchiolin.

Excision of ABO

Removal of the muricid ABO was performed as follows. The shell of the snail was cracked gently with a hammer over a brick without tearing the soft parts, and shell fragments were removed with for-

ceps. The snail was then cut in two at the base of the tentacles with scissors, thus separating the visceral from the pedal mass. The foot was pinned upside down to an eraser embedded in a wax-filled bowl, exposing the ABO pore. The ABO, including its short stalk, was excised with iris scissors and watchmaker's forceps under a binocular magnification of 10 times. In *Eupleura,* the ABO was usually everted by pressure of pinning and was readily removed, but in *Urosalpinx* the organ had to be cut out of its pedal sac (Carriker, 1943, Fig. 10). The operation was performed out of sea water to minimize the dilution of ABO secretion. The average diameter of relaxed ABOs used in these experiments in *U. c. follyensis* ranged from 1.8 to 2.6 mm, and in *E. c. etterae* from 1.5 to 2.0 mm. Because of the relatively small size of the organs and danger of rapid desiccation in air, they were transferred immediately from the snail to the substrates with fine forceps.

ABO-Substrate Preparations

The simplest preparation consisted of the excised ABO placed in a drop of filtered sea water (Millipore filter, pore size 1.2 microns) 2 to 3 times its diameter on an area of the calcareous substrate marked with lead pencil to facilitate subsequent localization of the site of the etching.

A deeper and more precisely circumscribed etching was often obtained by use of an artificial chamber, and the life of the excised ABO was extended by the use of antibiotics. We employed Millipore-filtered sea water containing 100 units of mycostatin and 250 μg of chloromycetin per ml. After being dipped in this solution the ABO was inserted in a short section of Intramedic polyethylene tubing with disk down and in contact with the substrate. This manipulation was performed under a magnification of 10 times with fine forceps. The diameter of tubing selected permitted the ABO to be housed snugly and the disk of the ABO to be supported squarely on the substrate. The latter was requisite since maximum etching was obtained directly under the ABO when the gland was in direct contact with the substrate. Additional sea water–antibiotics solution was added to provide a narrow zone of fluid around the outside of the chamber at the tube-substrate interface. Drops of the sea water–

antibiotics solution (pH 6.8) placed on shell as a control produced no disfigurement. Interposition of glass wool fibers between the ABO and the substrate produced a more diffuse pattern of dissolution. Application of the entire pedal region with everted ABO did not increase the degree of etching over that produced by excised ABO alone. ABO-substrate preparations were housed at room temperature in a covered glass or plastic chamber on wet cheesecloth to prevent drying. After an interval of 3 to 24 hours the ABO-chamber preparation was flushed from the substrate by a stream of distilled water, the substrate surface was air dried to prevent scratching, and the substrate was stored in dustproof containers. The time when the activity of the ABO ceases after excision is not known. At high summer temperatures the ABO starts to disintegrate after about 20 hours, but with the use of antibiotics the organ retains its form for a much longer period, and thus the pattern of etching can be related precisely to its creases and peripheral contour.

pH Determinations

Minute strips of short-range pH indicator papers, the color verified against standardized buffer solutions checked with a pH meter, were applied to specimens and compared with the color standards under the microscope.

ABO Homogenates

ABOs from 25 *U. c. follyensis* were excised rapidly, placed in two drops of sea water–antibiotics in an iced vessel, finely homogenized by grinding with fine sand, and diluted to a final volume 10 and 50 times that of the ABOs with the same sea water–antibiotics solution.

Artificial Etchings

These were produced by application of various chemical agents to portions of the valves of *C. virginica* and *M. fulvescens*. Shell surfaces were immersed for periods varying from 5 to 60 seconds in 0.1 N HCl (pH 1.0), 0.1 N lactic acid (pH adjusted to 5.0 with NaOH); for 2 minutes to 3 hours in boiling 60 per cent KOH; for 1 to 3 hours in 80 per cent ethylenediamine at 80°C; and for 24 and

48 hours in 10 per cent disodium versenate (pH 7.5–8.0) at room temperature.

Optical Microscopy, Electron Microscopy, and Microradiography

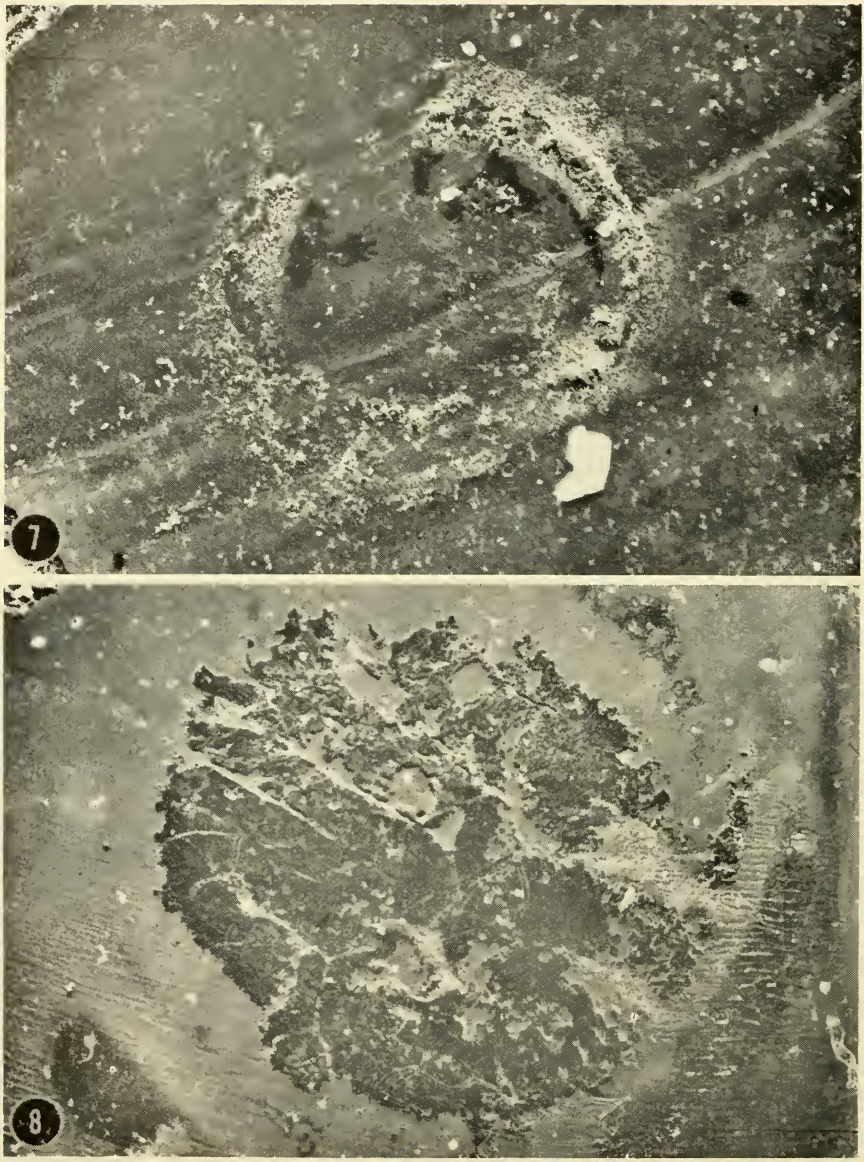
The methods described by Scott and Wyckoff (1946, 1956) were employed in the optical and electron microscopic study of etched substrate surfaces by means of shadowed replicas. Contact and projection microradiographs were made from sections of shells by the various technics outlined by Scott *et al.* (1962). Figures 5 to 49 in this paper are all negative prints; electron-opaque objects thus appear white, and shadows appear black.

Observations and Results

Activity of ABOs and ABO Homogenates

Repeated pH determinations of the fluid clinging to normally withdrawn and normally extended ABOs, excised ABOs, and ABO homogenates of muricid snails gave a neutral or slightly alkaline reaction, thus confirming similar observations of Ankel (1938), Fischer (1922), and Hirsch (1915). Early inconclusive tests with litmus paper by Schiemenz (1891) suggesting that the secretion is slightly acid were not corroborated. We found that the pH of fluids in incomplete bore holes was slightly alkaline, but this might be attributed to a buffering action of dissolved $CaCO_3$. The pH of the freshly prepared ABO homogenate ranged from 7.3 to 7.5 and remained so for some 20 hours. More refined determinations of the hydrogen ion concentration of the secretions of the ABO are in progress, but it is doubtful whether these will disclose values significantly different from those already obtained.

As viewed directly at low optical magnifications, the characteristic gross pattern of etching of shell surface produced by the excised ABOs took the form of a pronounced chalky white disturbance in the region immediately affected by the ABO, and a faint etching beyond this under the drop of sea water. In light etchings (Fig. 7) first damage occurred in a ring at the outer boundary of the ABO



FIGURES 7 AND 8

and again at the edge of the bubble of sea water. In deeper etchings (Fig. 8) the entire area under the gland and sea water was affected. Contact of the distal secretory cap of the ABO with the substrate produced the most active dissolution. Some of the demineralizing agent must have diffused into the sea water around the ABO and beyond the tube chamber to produce the peripheral etching (Fig. 16). Evolution of gas was not observed even at magnifications of 10 times during etching. That the etched area of shell is considerably more fragile than the surrounding shell was demonstrated by wiping a coarse tissue paper across the etched shell of *Murex fulvescens;* the paper left no mark whatsoever on the unaffected surface, but deeply scratched the damaged region. This fact is further illustrated by the trail of a lead pencil drawn across etched and nonetched surfaces in Fig. 18.

The demineralizing activity of ABOs of snails from mid-temperate latitudes was accelerated with increasing temperatures in the range of 5 to 30°C. However, ABOs heated approximately to 80°C for 2 minutes produced only very faint etched lines at the edge of the drop of sea water surrounding them. Etching of shell by excised ABOs began the moment the organ was placed on shell, and proceeded for a variable period. Marked etchings were obtained in as short a time as 3 hours. Although the majority of excised ABOs etched shell, there was generally a small minority which etched only slightly or incompletely, even under consistently uniform conditions. This suggests that there may be variation in the capacity of individual snails to etch or that secretion may occur periodically in association with the boring behavior; however, tests comparing the etching capacities of ABOs excised from actively boring and from resting snails have not been conclusive to date. Whether the organ continues

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Fig. 7. An optical micrograph made from a silver-shadowed negative replica of the nacreous surface of the adductor muscle scar of the clam *Dosinia discus* on which an excised ABO of a small moon snail, *Polinices duplicatus,* had been placed for 24 hours *in vitro.* Etching took place principally under the periphery of the ABO. (× 50.)

Fig. 8. A similar optical micrograph of the nacreous surface of the shell of *Murex fulvescens* on which an excised ABO of the naticid *Sinum perspectivum* had been placed for 24 hours *in vitro.* In this case damage to the shell substrate occurred beneath the entire ABO. (× 50.)

to produce additional secretion after excision, or whether only that already present at the time of excision is utilized, is not known. The variability in degree of etching in different preparations suggests the latter.

Excised ABOs held in distilled water for a minute or more prior to placement on the substrate produced very little or no etching, and immersion for a longer period evoked the formation of a dense halo of what appeared like secretory globules over the secretory disk of the ABO. These globules were not mucus, since histochemical tests disclosed no mucus cells in the secretory epithelium or in the subepithelial region of the ABO.

Excised ABOs rinsed in and placed in drops of saline buffer of pH 5.7, 7.4, 8.2, and 8.8 all produced etchings on shell. At the end of the 24-hour incubation period, the pH of all the fluids was consistently 7.5 or above. The degree of etching under the ABO at the initial pH of 5.7 differed from that under the ABOs at higher pH's, probably because of the influence of the acid buffer.

Most striking of all the tests performed with ABOs was the clear-cut demineralizing action of ABO homogenate diluted 10-fold. The pH of this ranged from 7.3 to 7.5. The most obvious etchings occurred as a pronounced ring at the outside edge of the drop of homogenate. A 50-fold dilution of the same homogenate also produced etching, although somewhat fainter, and also as a clear line around the outer edge of the drop (Fig. 18).

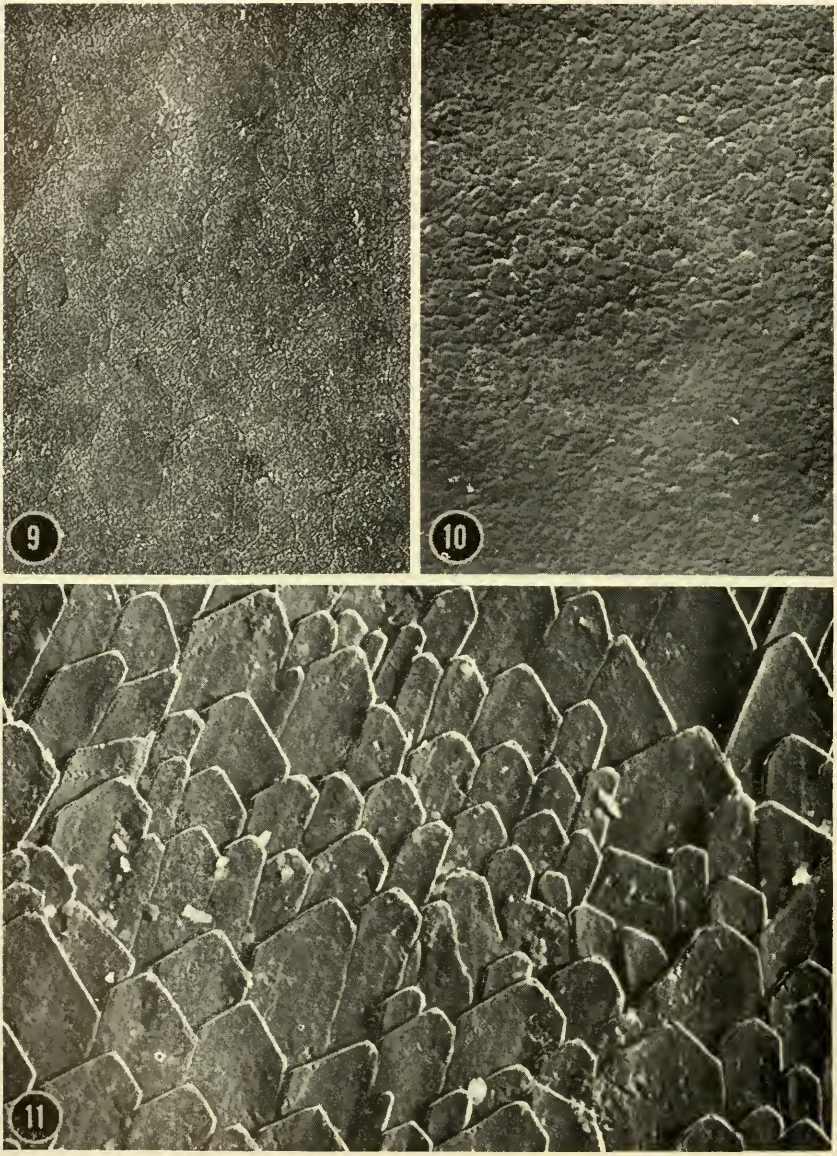
So far we have been unable to find any other tissues in the muricid snail body which can etch shell in the manner of the ABO. Minced and whole portions of pedal epithelium from various parts of the foot, salivary glands, accessory salivary glands, and glands of Leiblein produced no effect whatever on the nacreous shell of *M. fulvescens*. Graham (1941) applied whole accessory salivary glands to polished shell, and extracts of the glands to polished shell and to finely precipitated $CaCO_3$, and was likewise unable to obtain dissolution of the $CaCO_3$. The gastropod mantle, on the other hand, may be able to dissolve shell; Dugal (1939) notes the use of shell by *Mercenaria mercenaria* to buffer the product of anaerobic glycolysis, and Watabe (1954) and Watabe *et al.* (1958) have seen

evidence of shell dissolution by the mantle in electron micrographs of the nacreous surface of oysters.

Morphological Characteristics of Shell Subjected to ABO Activity

The appearance of complete muricid and naticid bore holes at low optical magnifications is shown in Figs. 1 to 4. When contact and projection microradiographs were made of bore holes in ground sections of shell 0.7 mm thick cut parallel to the original surface of the shell (Fig. 5), there was evident what appeared like a relatively wide partially demineralized region surrounding the periphery of the hole. This zone was even more apparent in projection microradiographs of sections of incomplete holes cut at right angles to the original surface of the shell (Fig. 6). That the density difference in the shell could not be accounted for on the basis of changes in contour of the hole through the section was established by use of stereoscopic projection microradiography. By this technic the contour of the surface of the hole in the shell section as well as the surrounding radiolucent zone were well demarcated and clearly distinguishable. We suggest that this partially demineralized band represents the result of ABO secretory activity which penetrated the shell to this degree.

Preliminary experiments were conducted to determine whether structural changes effected by ABO secretion in shell could be detected by means of surface replicas examined under optical and electron microscopes. Very shallow incomplete holes bored by *Urosalpinx* and *Eupleura* in the natural shell of living *Crassostrea virginica* in the laboratory were employed. Silver-shadowed negative collodion replicas were prepared for optical microscopy, and collodion-carbon positive replicas for electron microscopy. It was soon learned that although some microscopic detail could be observed on the surfaces of the borings themselves, excessive quantities of organic detritus and loosened shell material consistently interfered with the preparation of adequate replicas. Furthermore, the presence of the organic periostracal layer on the external surface of the shell surrounding the excavation prevented comparison of the altered



FIGURES 9 TO 11

shell surface in the bore hole with unaffected mineral-conchiolin shell surface around it. To obviate this difficulty we removed the periostracum and polished the external surface of shell of living oysters with a small electric sander and buffer. Snails readily bored into these oysters, but detritus still interfered with comparisons of shell surfaces; in addition, this experimental approach did not permit a clear distinction to be made between effects of etching by the ABO secretion and rasping by the radular denticles.

Because of the roughness of the external surface of most bivalve shells, and particularly those of oysters; because of the fineness of etching by the ABO and the difficulty of studying this on rough surfaces; and because to date it has not been possible to stimulate live snails to bore shell unless there is living prey within, it was necessary next to employ excised ABOs, disk down, on the smooth nacreous inner surface of mollusk shell. As described in the section on methods, we also subsequently used excised ABOs enclosed in cylinders of minute tubing, and drops of diluted ABO homogenate. Because boring gastropods appear to drill with equal ease through both nacreous and prismatic portions of calcitic and aragonitic mollusk shells, the inner surface was considered a representative substrate for these studies. Typical etchings produced by these means on different kinds of shells are shown in Figs. 7, 8, 16, and 18 at low optical magnifications.

Electron microscopic examinations were made of unaffected areas near the region on which the ABO had been placed and of the etched areas under the ABO. As illustrated in Figs. 9 and 10, the normal aragonitic nacreous surfaces of *Dosinia discus* and *Murex fulvescens* appeared quite smooth, whereas on the calcite nacreous surface of *Crassostrea virginica* (Fig. 11) very definite crystal outlines, similar

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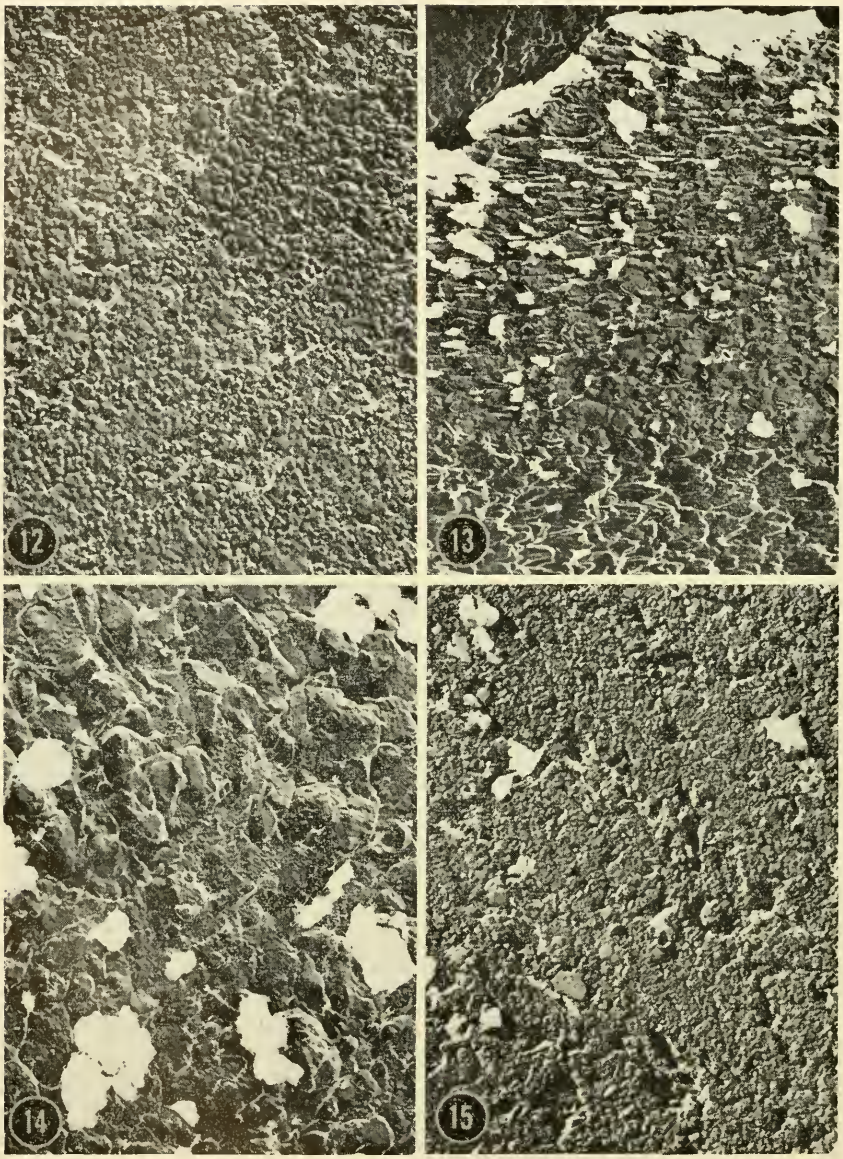
FIG. 9. Electron micrograph made from palladium-shadowed positive collodion-carbon replica of normal nacreous surface of *Dosinia discus*, showing a relatively smooth surface. (× 6000.)

FIG. 10. A similar micrograph of the normal nacreous surface of *Murex fulvescens*, showing very faint structural detail. (× 6000.)

FIG. 11. A similar micrograph of the normal nacreous surface of *Crassostrea virginica*, illustrating the characteristic and markedly crystalline nature of the surface. (× 6000.)



FIGURES 12 TO 15

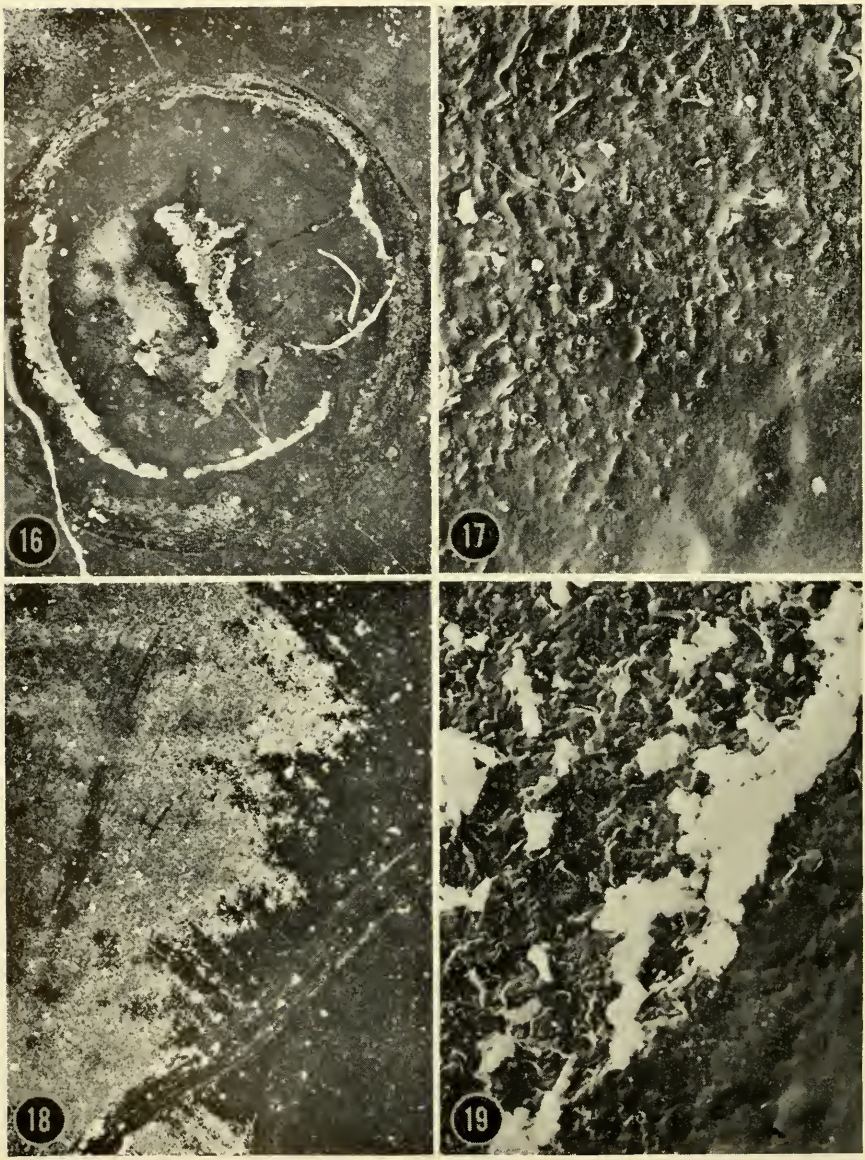
to those illustrated for the same species of oyster by Tsujii *et al.* (1958) and Watabe *et al.* (1958), were observed.

Structural alteration of the calcitic and aragonitic nacreous shell of three different species of bivalves and one species of gastropod by the secretion of ABOs from four different species of borers was similar so far as could be determined by inspection of the electron micrographs. Etched areas showed a variety of structural configurations, depending primarily upon the depth to which the superficial damage had progressed (Figs. 12 to 15, 17, and 19). Earliest evidence of surface alteration appeared as a slight generalized pitting. In more severely damaged regions the pits appeared larger and varying amounts of loosened mineral material were removed on the replicas. It was often necessary to take several replicas from a deeply etched area before enough of the loosened detritus was removed to provide a faithful reproduction of the firm underlying surface. At this level, widespread pitting and some crystal outlines were observed, but it was not possible to determine whether there was any preferential activity of the ABO secretion on the mineral or the organic constituents of the shell.

The most informative preparations proved to be the preliminary pseudo replicas, on which the adherent loosened crystal outlines could be examined in some detail (Figs. 20 to 25). The moth-eaten appearance of these crystals (see especially Figs. 21 and 25), as well as their serrated edges (see especially Figs. 20, 21, and 25) and the wide intercrystalline spaces, strongly suggests that these crystals underwent a marked degree of surface solution, and that loosening resulted from damage to the crystals rather than to the supporting organic matrix.

Figs. 12 to 15. Electron micrographs of areas of the nacreous surfaces of mollusk shells subjected to activity for 24 hours *in vitro* of ABOs placed directly on the specimens. The replicas from which these pictures were made represent the true surfaces after loosened debris had been removed by taking several preliminary replicas. Varying degrees of early pitting and roughening are shown. (× 6000.)

Fig. 12. ABO of *Urosalpinx cinerea follyensis* on *Murex fulvescens*.
Fig. 13. ABO of *Sinum perspectivum* on *M. fulvescens*.
Fig. 14. ABO of *U. c. follyensis* on *Dosinia discus*.
Fig. 15. ABO of *U. c. follyensis* on *Crassostrea virginica*.



FIGURES 16 TO 19

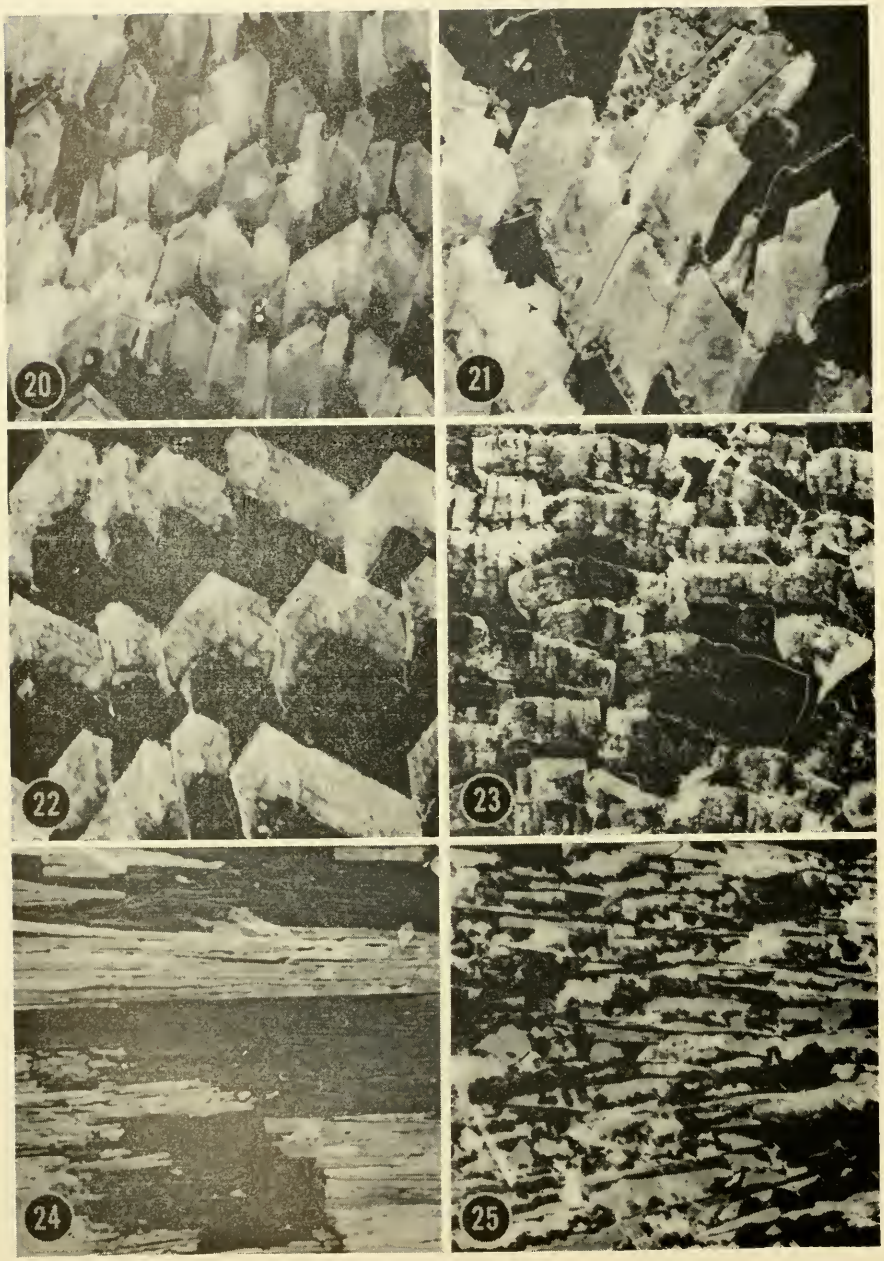
Morphological Characteristics of Shell Treated Artificially by Chemical Agents

As an aid in interpreting the etching patterns produced by ABOs we attempted to determine whether comparable alterations could be produced artificially through the treatment of (*a*) nacreous calcite shell surface (*C. virginica*) and (*b*) nacreous aragonite shell surface (*M. fulvescens*) with (1) agents which primarily attack the mineral portion, such as organic or inorganic acids, and a sequestering agent, versene, (2) agents which attack selectively the organic component, such as ethylenediamine, and (3) agents which attack both the organic and inorganic constituents, such as strong alkalies. Accordingly shell surfaces were immersed in (1) HCl or lactic acid and sodium versenate, (2) ethylenediamine, and (3) KOH.

Some typical examples of the surface effects of these various agents, as compared with that of the secretion from the ABO (Figs. 12 to 15), are shown in Figs. 26 to 31 for calcitic and aragonitic shells. Although none of the treatments resulted in damage exactly like that observed in the shells exposed to ABO activity (Figs. 12 to 15), disfiguration of the acid-etched (Figs. 26 and 27) and versene-treated (Figs. 28 and 29) specimens bore the closest resemblance. Particularly striking was the comparability of damage to the crystals themselves, such as pitting and serrated edges, caused by HCl and versene (Figs. 32 to 37) and by the ABO secretion (Figs. 20 to 25).

FIGS. 16 AND 17. Optical (Fig. 16, × 50) and electron (Fig. 17, × 6000) micrographs showing the etching produced by an ABO from *Urosalpinx cinerea follyensis* which was placed in a piece of minute plastic tubing against the nacreous surface of the shell of *Spisula solidissima*. The sharp impression of the outer edge of the plastic tube is clearly evident where it sank into shell material weakened by the ABO secretion. A lightly damaged area is illustrated in the electron micrograph.

FIGS. 18 AND 19. Optical (Fig. 18, × 50) and electron (Fig. 19, × 6000) micrographs showing the etching produced by a drop of the homogenate made from the ABOs of *U. c. follyensis*, diluted 50 times and placed on the nacreous surface of the shell of *S. solidissima*. A very heavily etched region is shown in the electron micrograph.



FIGURES 20 TO 25

Morphological Characteristics of Mineral Calcite and Aragonite Subjected to ABO Activity

The next step in these experiments was to define more precisely the properties of ABO secretion with regard to its capacity to attack the organic and inorganic components of shell. In order to determine the effect of ABO activity on the inorganic portion alone, we placed ABOs for 3 hours on flat cleaved surfaces of pure mineral calcite and aragonite crystals, which obviously contained no organic matrix. In such specimens definite surface alterations were observed (Figs. 38 to 43), and these resembled in large degree the changes which were earlier observed on shell (Figs. 16, 17, 12 to 15). Although the crystalline structure of pure calcite and aragonite is obviously different from that in mollusk shell, it is of interest that the earliest surface changes in the calcite and aragonite took the form of small pits (Fig. 40) which later coalesced to form much larger depressions (Fig. 43). The fact was thus established that the ABO secretion is capable of attacking the inorganic constituents of mollusk shells.

A comparable series of studies is now in progress in which isolated shell matrix is being subjected to similar activity. Considerable technical difficulty has been encountered and the work has not progressed far enough at this point to warrant discussion of the results.

Effect of the ABO Secretion on Mineralized Tissue Containing Calcium Phosphate

In order to characterize the ABO secretion further, we carried out an experiment to determine whether its activity is specific for calcium carbonate. Excised glands were placed on the polished outer

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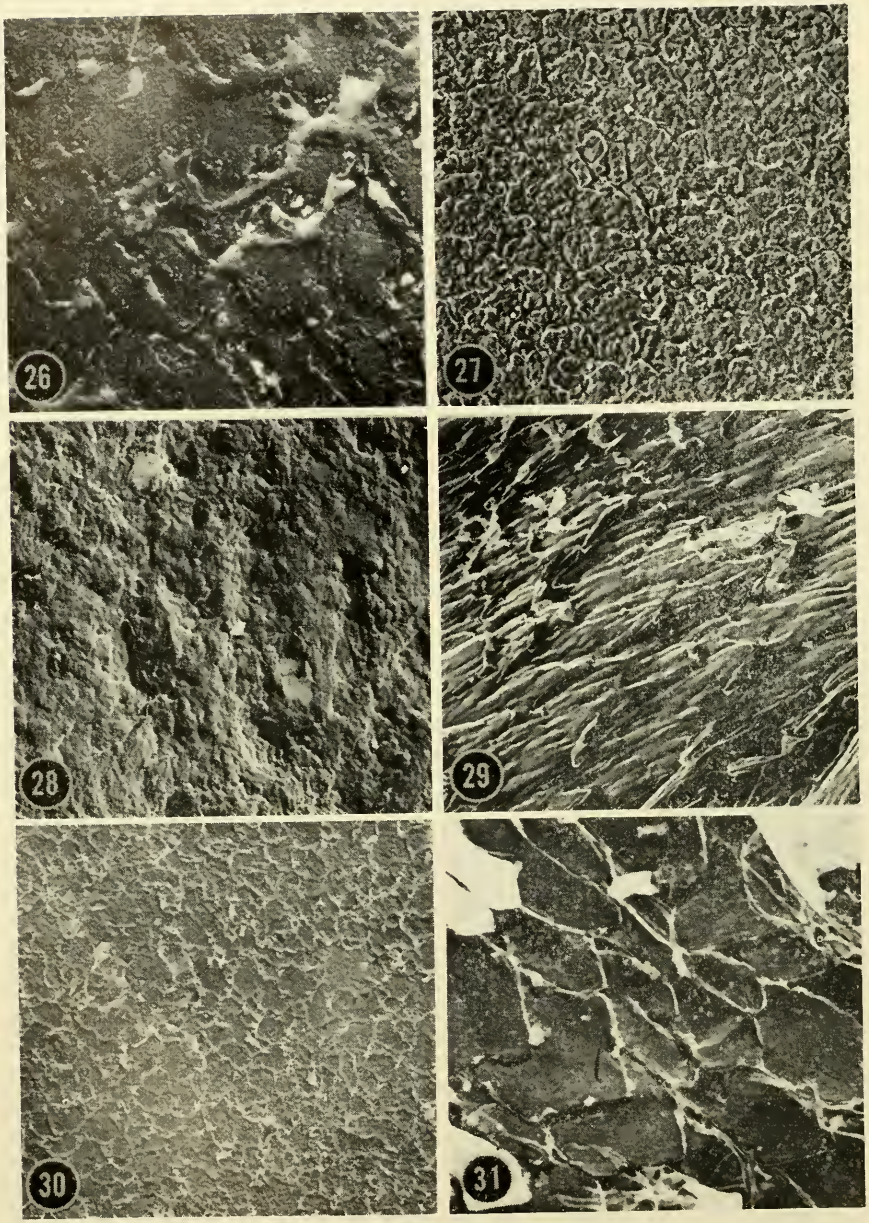
FIGS. 20 TO 25. A group of electron micrographs made from pseudo replicas on which thin layers of loosened crystalline material were removed from the nacreous surfaces of shells subjected directly to the action of excised ABOs. The pitted surfaces and serrated edges of the crystals are clearly shown. (× 6000.)

Figs. 20 to 23. ABO of *Urosalpinx cinerea follyensis* on *Crassostrea virginica.*

Figs. 24 and 25. ABO of *Polinices duplicatus* on *Murex fulvescens.*



FIGURES 26 TO 31

enamel surfaces of permanent tooth crowns and on the polished surfaces of ground sections of enamel and dentin. The mineral in tooth substance is calcium phosphate in the form of hydroxyapatite. It was found that both enamel (Figs. 44 to 47) and dentin (Figs. 48 and 49) were damaged by the glandular secretion. The rate of attack was somewhat slower than in the case of the shells, and it was necessary to prolong the treatment times to 60 to 100 hours by successive application of freshly excised ABOs at approximately 24-hour intervals. Although there is a small amount of carbonate (about 3 per cent) in tooth mineral, it seems unlikely that the degree and pattern of damage observed could be accounted for on the basis of carbonate solubility alone. These results suggest that the ABO secretion is probably not specific for calcium carbonate, but that at least calcium phosphate may also be attacked.

Discussion and Conclusions

Secretory activity of the accessory boring organ of drilling gastropods results primarily in a demineralization of the shell of prey, in the course of which mineral crystals appear to be partially dissolved and loosened. Dissolution of individual crystals appears to proceed irregularly, resulting not only in conspicuous intercrystalline spaces, but also in serrated edges and shallow to deep pits. Whether this differential rate of solution is due to the heterogeneous composition of each crystal, resulting in portions which are more vulnerable than others, or to an uneven distribution of the chemical agent, is not known. The former is more likely in view of the fact that HCl and versene produce grossly similar effects on these crystals. The degree

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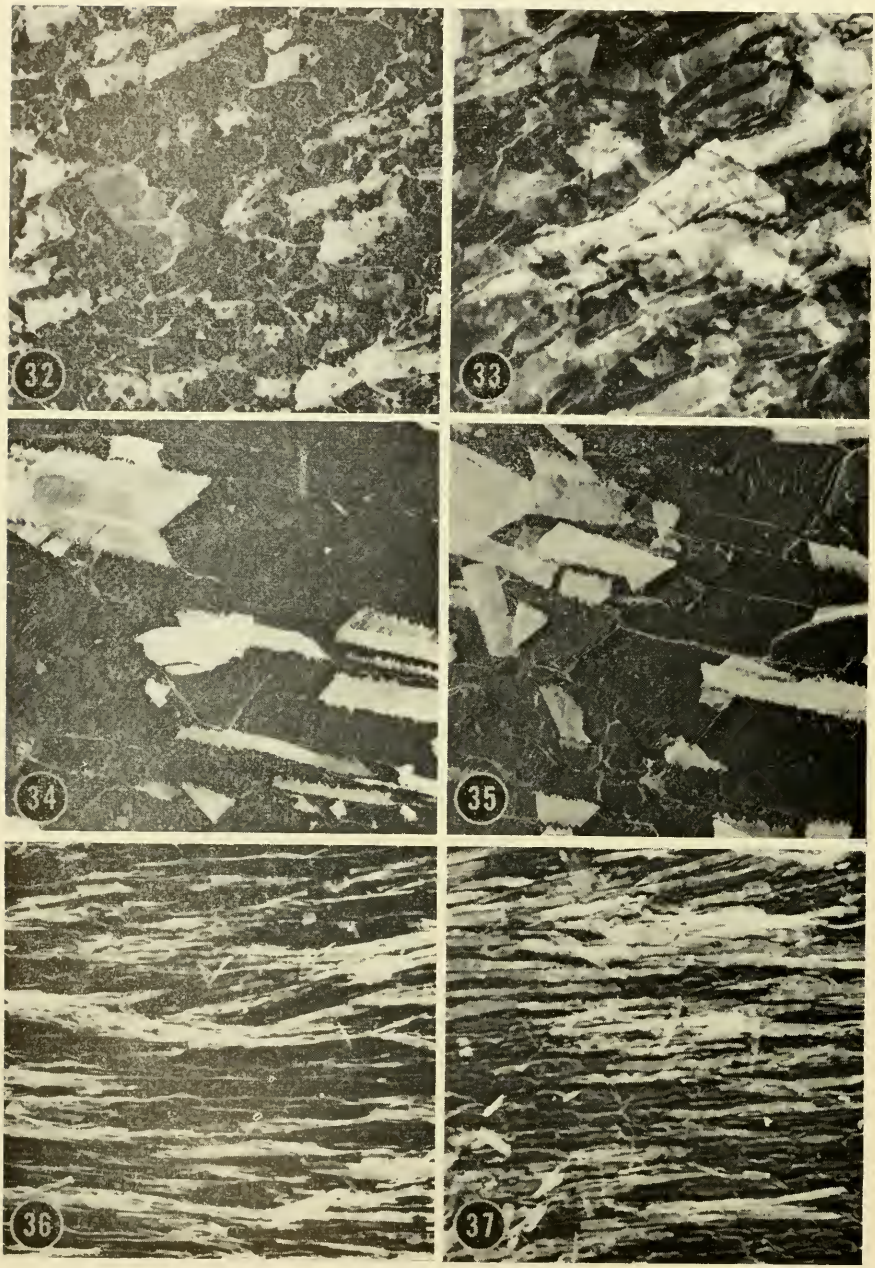
Figs. 26 to 31. Electron micrographs showing the character of the underlying nacreous surface following treatment with various chemical agents and removal of debris by preliminary replication. (× 6000.)

Fig. 26. *Crassostrea virginica*, 5 seconds in 0.1 N HCl, pH 1.0.
Fig. 27. *Murex fulvescens*, 5 seconds in 0.1 N HCl, pH 1.0.
Fig. 28. *C. virginica*, 48 hours in 10 per cent versene, pH 8.0.
Fig. 29. *M. fulvescens*, 24 hours in 10 per cent versene, pH 8.0.
Fig. 30. *M. fulvescens*, 3 hours in ethylenediamine at 80°C.
Fig. 31. *C. virginica*, 2 hours in boiling 60 per cent KOH.



FIGURES 32 TO 37

to which crystals may be loosened in one application of an active ABO was indicated by the successive number of replicas necessary to reveal the topography of more intact shell beneath. Action of ABO secretion on the organic matrix of shell, if any, is not yet known. Since radular teeth can rasp through the surface layer (or periostracum) of conchiolin on shell (Carriker, 1943) and through the noncalcareous horny wall of the egg capsule of a gastropod (Jensen, 1951), loosening of mineral crystals alone should be sufficient to permit effective boring by the snail. The duration of the period (from a few minutes to almost an hour) during which the ABO is extended by living gastropods on the shell of living prey is not inconsistent with the time required to obtain deep etchings by excised ABOs *in vitro*. Likewise significant here is an earlier observation (Carriker, 1943) that snails rasp in the hole for relatively short periods of time and generally only while shell flakes can be removed. If it should subsequently be found that the conchiolin support for the crystals is also weakened or destroyed, the removal of shell by the radula would be seen to be facilitated that much more.

The absence of acid in the secretion of the ABO and in ABO homogenates, the strong effect of the active agent on calcareous substrates, and the near inactivation of the agent when heated to 80°C give support to the suggestion of Hirsch (1915) that one constituent of this agent may be a kind of "calcase." Our studies also suggest that a chelating agent, or a chelating agent and enzyme combined, may be involved. The electron micrographs of nacreous shell surface etched by versene support the chelating agent hypothesis as clearly as does an electron micrograph by Grégoire (1957) of a polished transverse section of the nacreous shell of the snail *Turbo* etched with sequestrene. It should be emphasized that the similarity of electron micrographs of etchings by the neutral to slightly alkaline

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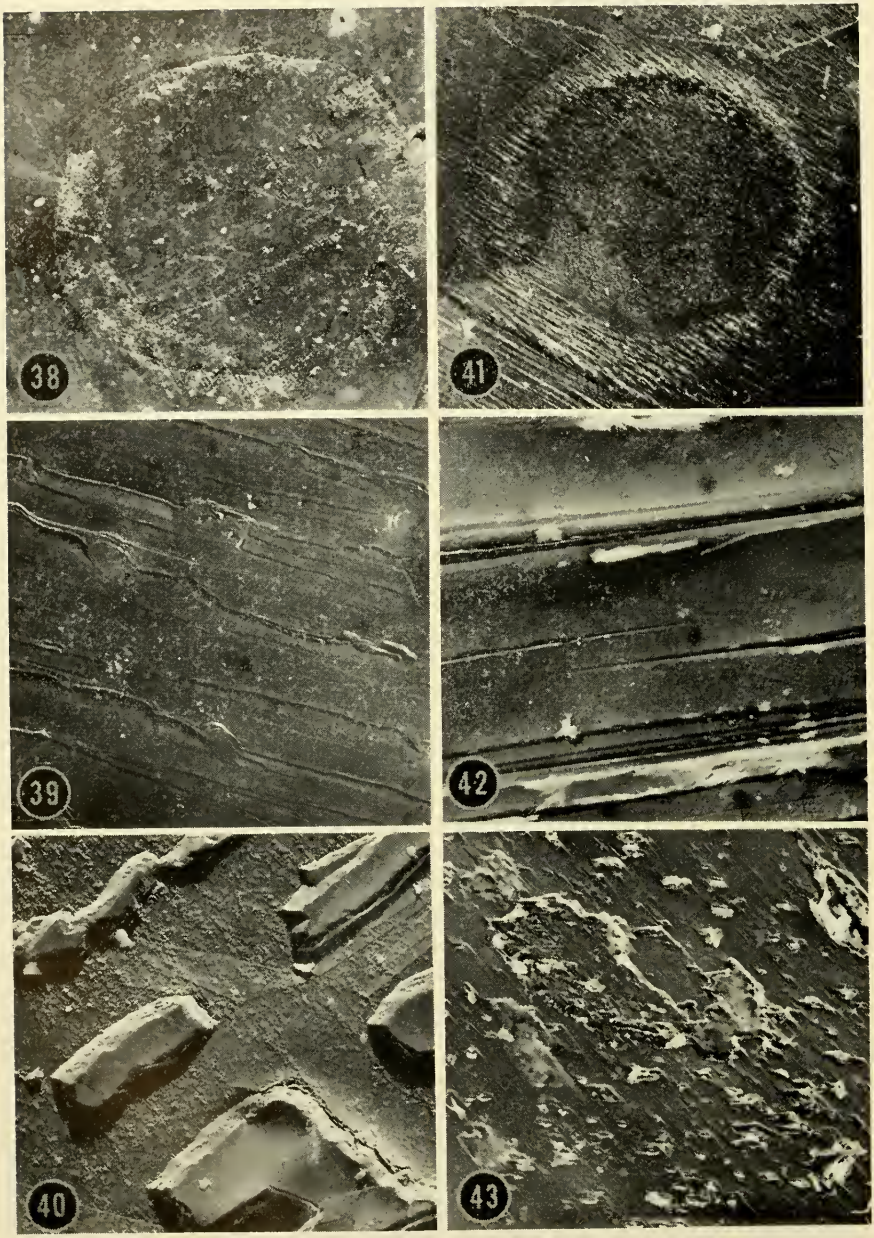
FIGS. 32 TO 37. A group of electron micrographs made from pseudo replicas on which loosened crystals were removed from the nacreous surfaces of shells subjected to the action of HCl and versene. Note the similarity of the damaged crystals to those in Figs. 20 to 25. (× 6000.)

Figs. 32 and 33. *Crassostrea virginica*, 5 seconds in 0.1 N HCl, pH 1.0.
Figs. 34 and 35. *C. virginica*, 48 hours in versene, pH 8.0.
Figs. 36 and 37. *Murex fulvescens*, 24 hours in versene, pH 8.0.



FIGURES 38 TO 43

ABO secretion and by acids suggests, not necessarily that the secretion is acid, but that alteration of shell surface by the two solutions is caused primarily by action on the crystalline portion of the shell.

The consistent uniformity and smoothness of gastropod bore holes has excited much interest. Results of studies reported in this paper and others in progress suggest that this uniformity is related to the interrelation of the morphology and functioning of the ABO and the proboscis tip. The secretory cap of the muricid ABO is located on a mushroom-shaped organ with a relatively long thin-walled stalk, and under hemostatic pressure the organ is extended fully and firmly from its sac in the mid-ventral anterior region of the foot into the bore hole. This brings the secretory epithelium of the ABO into close contact with the bottom of the hole and confines the active agent principally to shell immediately under the ABO disk, producing etchings similar to those observed in *in vitro* ABO-substrate preparations. Close appression of the thin walls of the stalk of the ABO to the sides of the bore holes blocks diffusion of most of the secretion to these walls. It is improbable that sea water moves into the bore hole as the snail alternates use of the ABO and the radula, since the hole is completely covered by the foot while the ABO is functioning, and in shifting to use the radula the snail extends its proboscis to the boring site between closely juxtaposed propodial folds. But if sea water does enter, it could be pressed readily from the hole by full extrusion of the ABO into it. The snail rasps principally at the bottom of the hole, rotating the radula to effect uniformity in penetration. Zones or areas of "harder" shell occasionally

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FIGS. 38 TO 43. Effects of secretion from excised ABO on pure mineral crystals.

Fig. 38. Optical micrograph of etching of ABO of *Eupleura caudata etterae*, 3-hour exposure, on calcite. (× 30.)

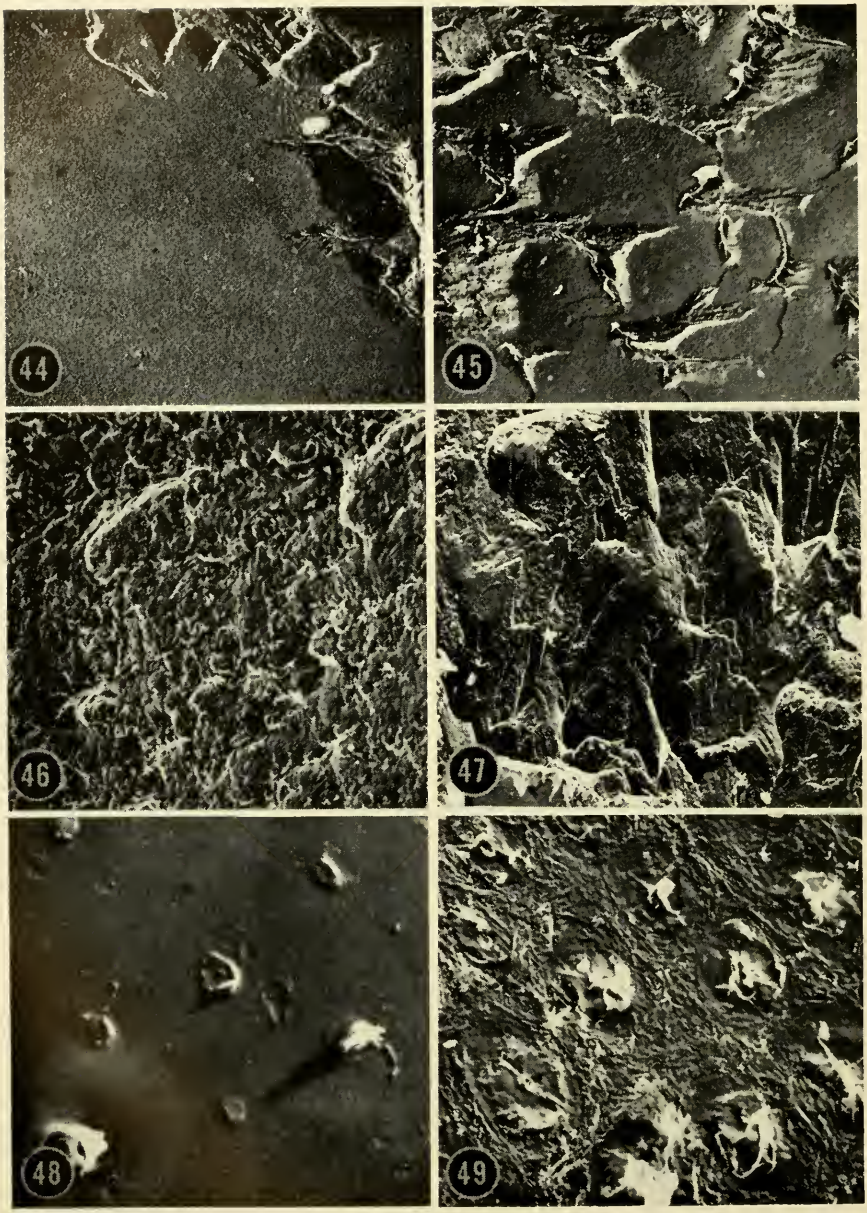
Fig. 39. Electron micrograph of normal calcite surface. (× 6000.)

Fig. 40. Electron micrograph of part of etched surface shown in Fig. 38. (× 6000.)

Fig. 41. Optical micrograph of etching of ABO of *Urosalpinx cinerea follyensis* after 3-hour exposure on aragonite. (× 30.)

Fig. 42. Electron micrograph of normal aragonite surface. (× 6000.)

Fig. 43. Electron micrograph of part of etched surface shown in Fig. 41. (× 6000.)



FIGURES 44 TO 49

disfigure this uniformity. Whether such regions contain greater concentrations of conchiolin which slow penetration of the ABO secretion, or interfere with rasping, is not yet known. The fact that shell softening occurs chiefly at the bottom of the hole and that the radula rasps principally also at this surface accounts for the fact that the muricid bore hole has relatively straight sides (Figs. 1 and 2). That some secretion also penetrates the walls of the hole is strongly suggested by the microradiographs (Figs. 5 and 6). The presence in most of these perforations of a very slight bevel at the external surface of the shell suggests that some of the ABO secretion spreads beyond the hole under the foot, permitting the radula to remove shell there too.

Because of the closeness of the propodial folds around the functioning naticid proboscis tip and appended ABO, it is unlikely that much, if any, dilution of the ABO secretion occurs when the proboscis is everted. The naticid ABO, though at least as efficient as the muricid ABO in softening shell, differs from the latter in being a padlike organ which produces a broadly beveled hole (Figs. 3 and 4), probably because it cannot be extended as precisely and fully into the concavity as the muricid ABO. In this sense naticid boring is less economical than that of muricids, since more shell must be removed to effect an opening of a given diameter at the bottom of the hole.

The action of the ABO secretion of different species of boring gastropods tested to date appears to be similar, not only on a given species of mollusk shell, but on the different species employed. The variation in ultrastructural topography observed on different etchings is a reflection of the degree of alteration of the shell rather than of species differences in the ABO or the shell substrate.

FIGS. 44 TO 49. Effects of secretion of excised ABO of *Urosalpinx cinerea follyensis* on polished surfaces of ground sections of human teeth exposed to 3 successive periods of ABO activity totaling 60 hours. (× 6000.)

Figs. 44 and 45. Sharp demarcation between affected and normal enamel.
Figs. 46 and 47. Progressively increasing damage to enamel.
Fig. 48. Normal dentin.
Fig. 49. Surface of dentin affected by ABO secretion; the more pronounced tubular, peritubular, and intertubular structures are evident.

Whether the mechanism is common to all boring gastropods will not be known until many more species around the world have been investigated.

Summary

1. Studies of the demineralization-boring mechanism of drilling naticid and muricid gastropods were continued. The following species of boring snails were investigated: *Urosalpinx cinerea follyensis, Eupleura caudata etterae, Murex fulvescens, Murex brevifrons, Polinices duplicatus,* and *Sinum perspectivum.* The following substrates were utilized: shell ($CaCO_3$ as calcite) of the bivalves *Crassostrea virginica, Dosinia discus,* and *Spisula solidissima;* shell ($CaCO_3$ as aragonite) of the snail *Murex fulvescens;* human enamel and dentin (calcium phosphate as hydroxyapatite); and pure inorganic calcite and aragonite ($CaCO_3$) without organic matrix. Bore holes were made by living gastropods in the laboratory; and etchings on substrates were produced by the secretion of the excised accessory boring organ (ABO) and ABO homogenates on special ABO-substrate preparations. These etchings were studied by microradiography and by optical and electron microscopy.

2. Repeated pH determinations of the fluid on normal ABOs, excised ABOs, and ABO homogenates gave a neutral or slightly alkaline reaction. The active agent of ABOs heated to 80°C was almost entirely inactivated.

3. Activity of the secretion of the ABO resulted primarily in a demineralization of the shell of prey; in the course of this, mineral crystals were partially dissolved and loosened for ready removal by radular denticles. Action on the organic matrix is not yet known, though it may be less readily attacked than the mineral constituents of shell. Both inorganic calcite and aragonite were soluble in glandular secretion; but the secretion was not specific for $CaCO_3$, as it acted also on calcium phosphate.

4. The action of the ABO secretion of different species of boring gastropods tested to date appeared to be similar, not only on a given species of mollusk shell, but on the different species employed. Variations in the ultrastructural topography of different etchings

was a reflection of the degree of alteration of the shell rather than of species differences in the ABO and the shell substrates.

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4

Dental Hard Tissue Destruction with Special Reference to Idiopathic Erosions

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MECHANISMS of hard tissue destruction within the jaws and teeth involve the full spectrum of vertebrate hard tissues: enamel, dentin, ivory, cementum, and bone. Biological elements that are presumed to be directly or indirectly responsible for the destructive processes range from the minute bacteria of dental decay to the large osteoclasts of tooth and bone resorption. In addition there are involved various physical and chemical agents from endogenous as well as exogenous origins, some known (masticatory wear and acidic juices), others hitherto poorly understood (idiopathic and saprophytic erosions). The latter will be the principal focus of interest in this discussion.

The situations in which the dento-alveolar hard tissues are destroyed range from normal physiological phenomena of growth, eruption, shedding, and aging to miscellaneous pathological processes of systemic and local etiology. Furthermore, as will be amplified later, these various processes of hard tissue destruction are not entirely one way, there being evidence of redeposition of calcified matter at certain stages. Finally, even if teeth and jaws (notwith-

standing modern man's widespread dental ills) are spared from destruction during life, subterranean saprophytes in search of food are waiting in the ground to consume the buried dento-alveolar organs by a biological capacity to dissolve both the solid inorganic salts, mainly calcium phosphate, and the proteinaceous organic matrices, mainly collagen ("The houses of ivory shall perish . . . ," Amos 3:15) (Sognnaes, 1960*c*).

The Spectrum of Dento-Alveolar Destruction

All told there are some thirty conditions, including those caused by growth, aging, pathology, therapy, and experimental procedures, in which the hard tissues of the dento-alveolar apparatus can undergo some form of biologically reversible or irreversible dissolution (Table I).

TABLE I. Dento-Alveolar Aspects of Hard Tissue Destruction

Bone destruction (maxillary and mandibular resorption processes)

1. (*a*) Growth, eruption, and aging
2. (*b*) Shedding mechanism
3. (*c*) Traumatic occlusion
4. (*d*) Orthodontic therapy
5. (*e*) Periodontal disease
6. (*f*) Metabolic bone disease
7. (*g*) Jaw tumors, cysts, and infections
8. (*h*) Radiation injury

Tooth destruction (enamel, dentin, and cementum)

I. *Dental attrition*

9. (*a*) Masticatory wear
10. (*b*) Traumatic, general, and localized

II. *Dental resorption*

A. Internal pulpal (dentin)

11. (*a*) Normal shedding
12. (*b*) Pathologic granulations ("pink tooth")

B. External periodontal (cementum)

13. (*a*) Shedding and aging
14. (*b*) Periodontal disease
15. (*c*) Periapical tumors, cysts, and infections
16. (*d*) Orthodontic therapy

[*Continued on following page*]

TABLE I—*Continued*

TOOTH DESTRUCTION—*Continued*

II. *Dental resorption—Continued*

B. External periodontal—*Continued*

17. (*e*) Traumatic occlusion
18. (*f*) Radiation injury

III. *Dental caries*

19. (*a*) Enamel
20. (*b*) Dentin
21. (*c*) Cementum

IV. *Erosion-abrasion*

A. *In vivo* destruction

22. (*a*) Known exogenous chemical agents (acidic juices, etc.)
23. (*b*) Known endogenous chemical agents (gastric acid, etc.)
24. (*c*) Chemical and abrasive combinations
25. (*d*) Therapeutic agents
26. (*e*) Experimental agents
27. (*f*) Idiopathic erosions
    (i) Wedge- and disc-shaped
    (ii) Irregular and figured
    (iii) Circumscribed

B. *In vitro* destruction

28. (*a*) Experimental physical
29. (*b*) Experimental chemical

C. Postmortem destruction

30. (*a*) Irregular boring canals
31. (*b*) Discrete surface "lacunae"

Dental Attrition

Normal masticatory function and aging are accompanied by occlusal and interproximal wear of the teeth. There ensues a shortening of the anatomical crowns of the teeth as well as of the dental arch so as to reduce the relative force of stress and strain on the root socket attachment to the alveolar bone.

Excessive wear can occur on individual teeth that are out of normal position or exposed to local trauma due to habits of chewing (pipestem wear, etc.) or on the whole dentition if exposed to abnormal general wear due either to unusual food habits or to diurnal or nocturnal bruxism. Even with regular function and food habits, rapid wear of the teeth can take place in the absence of the lubricat-

ing action of the saliva (an 18-year-old girl with complete xerostomia was once seen whose mouth was paper dry, and who had worn her teeth down to the gum line, so that she had the appearance of an old Eskimo).

Mention should also be made of the extremely destructive wear that may occur as a result of the combination of mechanical and chemical influences; that is, accelerated attrition of tooth substance partly weakened by chemical dissolution. The most interesting example of such combinations is the excessive wear reported in sheep's teeth (Barnicoat, 1957, 1960, and this volume, chapter 5) which has been attributed not only to masticatory wear, but also to the action of organic acids from herbage, in combination with freshly expressed juices of grasses and clovers containing enzymes capable of acting on the organic portion of the teeth (proteolysis). In other words, it is suggested that the rapid wear of dentin in sheep may be due in part to attrition, in part to demineralization, and in part to digestive action on the organic matrices by the proteinases of actively metabolizing leaves.

Alveolar Bone Resorption

When the process of resorption affects the maxillofacial bones, it differs in no conspicuous histomorphological way from the typical lacunar resorption seen elsewhere in the vertebrate endoskeleton. Multinucleated giant cells are characteristically found in typical Howship's lacunae along the resorbing bone surfaces, be it in normal alveolar remodeling accompanying growth, eruption, and shedding (see chapter 12 by Bhaskar), in pathological bone destruction of periodontal disease (see chapter 11 by Reichborn-Kjennerud), or in modified metabolic states such as hibernation (see chapter 10 by Mayer and Bernick); or in other parts of the skull, beyond the dento-alveolar apparatus, e.g. in experimentally induced calvarium destruction, be it in living animals (see chapter 17 by Young), in *in vitro* explants (see chapter 23 by Dowse *et al.*), or in tissue culture (see chapter 24 by Goldhaber).

Within the supporting tissues of the teeth, however, special mention should be made of several phenomena of local significance and general interest.

Firstly, the resorption of alveolar bone during growth and eruption of the teeth, which usually occurs at the bone surfaces subjected to physiological pressure, is synchronized with processes of bone apposition at opposite zones of tension. Thus teeth are kept in functional balance during long periods of growth, eruption, and alveolar bone remodeling. Indeed, it is in part this very biological fact, rather than mechanical appliances per se, which makes possible orthodontic tooth movement in definitive directions (Sognnaes, 1955, 1960*a*, 1960*b*).

Secondly (and this is the other biological blessing to which orthodontics owes its very existence), the dental root cementum—albeit within close neighborhood of alveolar bone, and notwithstanding that its constituents are almost identical with those of bone—is remarkably well protected from resorption. This is presumably due to minor but yet adequate differentials in the immediate vascular and cellular environments.

Thirdly, in the process of normal shedding of the teeth, the alveolar bone sockets (as well as all the specialized dental tissues, cementum, dentin, and even enamel) undergo extensive localized resorption from one jaw region to another, one tooth after another (in man, 20 deciduous teeth) over a long period of time (in man, from 6 to 13 years of age) seemingly unrelated to any general systemic control, in contrast to the simultaneous, sex hormone–controlled shedding of antlers (see chapter 13 by Goss).

Fourthly, in the presence of metabolic disease which otherwise tends to affect the human skeleton, the alveolar jaw bone may in some conditions be among the very first to show signs of destruction (lamina dura resorption in hyperparathyroidism and "loose teeth" in scurvy), or in other conditions may be relatively unaffected in the presence of severe generalized bone disease, as in the case of osteoporosis (see chapter 15 by Urist *et al.*).

Fifthly, although it is the primary responsibility of the dentist—assisted by routine roentgenological examinations—to recognize rarefying jaw lesions of dental origin (abscesses, cysts, and odontogenic tumors), there are numerous nondental conditions which may cause extensive resorption of the jaw bones, including nondental local and metastatic tumors, as well as primary and secondary ef-

fects of radiation brought about by accidental (radium dial painters) or therapeutic (cancer treatment) exposures.

Dental Resorption

The dental hard tissues are subject to bonelike resorption processes in the presence of osteoclast-like multinucleated giant cells situated in characteristic Howship's lacunae along resorbing surfaces of cementum, dentin, and, in some cases, even enamel. Of general significance in this connection are, on the one hand, the environmental and systemic conditions under which these resorptive conditions occur, and, on the other, the range of composition or nature of the resorbing tissues themselves (Sognnaes, 1955, 1960*b*). Typical examples of resorption within the dental apparatus are described elsewhere (see Bhaskar, chapter 12, and Reichborn-Kjennerud, chapter 11); we are here specifically asking ourselves what light if any the dental aspects of this process may shed upon the fundamental explanation of resorbability per se.

In the first place, it is noteworthy, in the case of *dental* resorption, that the origin of the multinucleated osteoclasts (perhaps more appropriately called "odontoclasts") cannot be attributed to the fusion of antecedent uninucleated cells within the tissues undergoing resorption. Neither enamel, dentin, nor primary cementum contains interstitial cells.

In physiological shedding of the teeth we have shown typical lacunar giant cells in juxtaposition to the resorbing dental enamel (Sognnaes, 1959, 1960*b*), a tissue completely devoid of even protoplasmatic extensions of any antecedent cellular elements. The dentin, which undergoes typical lacunar resorption both in normal shedding and under pathological conditions of internal resorption ("pink tooth"), has no connective tissue cells within its matrix, albeit permeated by long cytoplasmic processes from the pulpal odontoblasts. In the case of cementum there are to be sure cells embedded in the widest periapical zone of secondary cementum (cementocytes), but the primary cementum is completely acellular and yet in no way spared from the same resorbability in the presence of typical multinucleated giant cells which obviously must originate from the adjacent environment (e.g. enamel resorption, see Fig. 30e).

As a second aspect of general significance, there appears to be no common link between the conditions under which these generally protected dental hard tissues succumb to resorption. In no case—even in physiological shedding—can one definitely point to a generalized systemic influence, such as is found in the case of deer antler resorption, where a definitive sexual cycle is involved (see chapter 13 by Goss). Unlike antlers, the teeth of man resorb one after another, summer and winter, over a long period of time between the ages of 6 and 13. But from a local environmental point of view, the shedding teeth share with the shedding antlers a juxtaposition to a highly vascularized pad of granulation tissue studded with giant cells which clearly must originate from the adjacent connective tissue environment. Even in pathological tooth resorption—be it internal (pulpal) or external (periodontal), of local origin or caused by metabolic bone disease (hyperparathyroidism), traumatic occlusion, or radiation injury—one typically finds proliferation of highly vascularized granulation tissue loaded with giant cells in immediate juxtaposition to the resorbing cementum, dentin, or enamel, as the case may be.

Finally, there are certain important differences in the structural and chemical nature of the three dental hard tissues, cementum, dentin, and enamel, which may help to clarify the question as to whether or not the basic phenomenon of resorbability is dependent to any significant degree upon the quality of the structures being resorbed.

In general, it may be said that the prevailing concepts of hard tissue biology hitherto have been largely evolved from studies of bone tissue characterized by minute inorganic crystals and a collagenous organic framework—whether the concepts deal with the process of resorbability or of calcifiability (Sognnaes, 1960*a*). But in the dental apparatus we are faced with a less than satisfactory explanation for the deposition and removal of the large inorganic crystals contained within the inadequately identified organic matrix of enamel, a product of ectodermal cells, recently classified as a cross-β-linkage protein (Glimcher *et al.*, 1961). At best this is a sparsely distributed organic framework. Thus, irrespective of its chemical nature, the quantity is so low as compared with the or-

ganic matter in other resorbable tissues that one is almost forced to disregard the basic role of inherent organic components within the tissues being resorbed—as we have already disregarded the role of interstitial cellular components—in the basic process of resorbability.

In conclusion, then, it is believed that the general significance of dental resorption phenomena rests on the observation that resorbability must be largely controlled by the consistent presence of a highly vascularized adjacent tissue environment rather than by some peculiarly consistent feature characterizing the structures that are being resorbed, whether these be enamel, dentin, ivory, cementum, or bone, in various states of calcification or with various chemical and physical characteristics of the organic and inorganic constituents (Sognnaes, 1955, 1960*a*, 1960*b*).

Dental Caries

The problem of dental caries, the most universal of all human ills, is being examined in several other chapters of this volume, from the point of view of microstructure (Darling, chapter 6), ultrastructure (Johansen, chapter 7), chemistry (Gray and Francis, chapter 8), and bacteriology (Keyes and Jordan, chapter 9).

As a preamble, however, to the principal theme of the present chapter—*in vivo*, *in vitro*, and postmortem erosions—it seems important to recognize several characteristic features of dental caries which are in sharp contrast to other types of hard tissue destruction in general and to other types of dental pathology, notably dental erosion, in particular (Sognnaes, 1959, 1962).

In the first place, dental caries is characterized by a deep subsurface demineralization (up to 1000 microns deep) prior to collapse of the tooth structure (i.e. prior to extensive proteolysis and "cavity" formation). Secondly, the pathway of this process is intimately related to certain preformed structural patterns within the dental hard tissues themselves. These are, in the enamel (*a*) the incremental lines of Retzius, (*b*) the interprismatic substance, and (*c*) the intraprismatic cross striations of the individual enamel prisms (these zones are probably related; Sognnaes, 1949); and in the dentin (*a*) the normal growth rings of von Ebner, (*b*) the Owen's contour lines (probably representing less than adequate calcification

to begin with), and (*c*) the pathways of the dentinal tubules, which contain protoplasmatic extensions from the pulpal odontoblasts and may be subject to secondary calcifications, decalcifications, and bacterial invasion as a late feature of caries.

As will be noted below, these manifestations of tooth destruction in caries are significantly different from those occurring in dental erosion *in vivo* and *in vitro*, on the one hand, and those occurring during postmortem destruction of the teeth, on the other.

Dental Erosion

There are numerous reports in the literature regarding erosion of teeth caused by various chemical agents with and without concurrent abrasion caused by mechanical friction.

Stafne and Lovestedt (1947) have observed a number of cases in which dissolution of tooth substance was caused by various kinds of acids, juices, soft drinks, etc. Similar effects have been observed as a result of certain medicaments (James and Parfitt, 1953), therapeutic use of dilute hydrochloric acid (Stafne, 1933), general hydrogen ion concentration (Elsbury, 1952), long-continued vomiting accompanying obstipation (Bargen and Austin, 1936), diabetes insipidus (Finch, 1957), excessive consumption of acidified candies (West and Judy, 1938), miscellaneous natural juices (Gortner and Kenigsberg, 1952), acidified beverages (McClure, 1943; McCay and Will, 1949; Restarski *et al.*, 1945; Gortner *et al.*, 1945), various types of fruit drinks (Miller, 1950; Holloway *et al.*, 1958), dietary oxalate (Gortner *et al.*, 1946), citrate and lactate (McClure and Ruzicka, 1946). These reports also contain observations on partial control of such erosions by various buffering agents, fluoride, etc. (Restarski *et al.*, 1945; Holloway *et al.*, 1958) as well as many cross references to other literature not cited here.

The degree to which abrasive action is involved in the progress of erosion-like lesions of the teeth is not fully understood. As a rule the lesions referred to above have the appearance of rather diffuse dissolution, whereas typical abrasion and atypical erosion (idiopathic, see below) may have a more definitive pattern depending upon the location of the frictional and/or chemical influences.

A most interesting pattern of erosion-like defects has recently been reported by Rost and Brodie (1961), whose observations suggest that mechanical friction without any chemical action had caused localized destruction, otherwise more typical of erosion. For example, in several cases where amalgam or gold fillings had been placed for the express purpose of repairing such lesions, the eroded pattern was subsequently found on the fillings themselves. In one case the erosion lines were found within 8 months on the surface of a plastic (methyl methacrylate) filling which had been placed in the center of the labial surface of an upper central incisor for the repair of a small congenital defect. In other cases delicate lines could be seen in the plastic base of dentures. The authors suggested that a possible cause of these lines might be some "hyperactivity" of the soft tissue environment of the structures involved, whether natural or artificial, and that such environmental abrasion might be one of the causes of the lesions observed. Since the plastic material would be resistant to any chemical action encountered in the mouth, one must conclude—in the opinion of this writer—that here are cases in which physical forces have been at work, possibly a twitching of soft tissue elements rubbing against the plastic material or perhaps even streams of saliva continuously running like miniature rivers in confined areas.

There can be no question that the hard tissues of the mouth are susceptible to destructive actions of mechanical or chemical nature or both. Hence one must expect to encounter mechanical abrasion and chemical erosion as well as combined erosion-abrasion of dual etiology. Calcium phosphate is obviously soluble in calcium-binding chemical agents, whether these be of exogenous or endogenous origin, as long as the agents are present sufficiently long, in adequate quantities, and in susceptible locations. Thus it is not surprising, as noted above, that chemical erosion of rats' teeth occurs after prolonged ingestion of what in human equivalents would be enormous quantities of acidic beverages. Similarly, it is to be expected that frictional forces will wear away the dental hard tissues. Perhaps it is more surprising that the dental organs can in fact last the prolonged lifetime of modern man without being either dissolved or

worn away in the face of the many *known* injurious influences within the oral environment.

There is, however, a more obscure type of dental hard tissue destruction that needs to be discussed in greater detail, namely the peculiar wasting of tooth substance which, for the moment, may be best classified as "idiopathic erosion." The following subsections will consider this problem from the point of view of (1) gross distribution, (2) microradiography, (3) histopathology, (4) tooth structure, (5) oral environment, (6) general constitution, and (7) clinical management.

Gross Distribution

The commonly held concept that dental erosion may be largely attributed to mechanical friction from tooth brushing became widely accepted following a series of studies by W. D. Miller, published in 1907. He based his conclusion on the observation that dental erosion is much more common in modern than in ancient man. Out of a total of 36,000 ancient skulls surveyed, none were said to have shown typical lesions of dental erosion, whereas among twice as many contemporary individuals dental erosion was found, and was claimed to be confined to those who had become introduced to the use of a toothbrush.

In retrospect, it is somewhat surprising how widely this circumstantial evidence was to be accepted, conflicting clinical experience notwithstanding. Indeed, even earlier writings on the subject had emphasized that the topographic configuration and distribution of dental erosion frequently failed to conform to the destructive characteristics that would be expected from mechanical friction of a toothbrush. Figure 1 illustrates this point. Some of these photographs are reproductions of plaster casts from patients seen in the private practice of Dr. Maurice Peters, who, in addition, has very kindly provided some of the specimens that have been further explored in my microscopic follow-up studies reported below.

It will be noted from the nine cases shown in Fig. 1 that a considerable variation can exist in location and configuration of the lesions. In some instances the lesions are located near the incisal

FIG. 1. Variability in the gross morphological distribution of dental erosion: A, B, narrow horizontal grooves; C, D, E, shallow, flat, and disc-shaped; F, irregular horizontal and vertical erosion separated by intact tooth; G, figured; H, I, circumscribed, wedge-shaped, labial (H) and lingual (I). For details see text.

edge, in others near the gingival margin, in others at both sites. In some mouths there are combinations of lesions running horizontally and vertically, and yet separated by completely unaffected teeth (Fig. 1F). This would be extremely difficult to account for on the basis of tooth brushing. Occasionally extreme lesions can be seen lingually in the gingival area of the lower anterior teeth, a location completely incompatible with a tooth-brushing etiology. In one such case, shown in Fig. 1I, the erosion ultimately progressed so far that the destruction circumscribed the whole cervical area, both lingually, interproximally, and labially, so as to produce an hour-glass appearance interestingly similar to a natural rock "erosion" (see Fig. 2 with insert).

The several examples of dental erosion shown in Fig. 1A to I conform (from the point of view of gross topography) to the classical description of G. V. Black, as retained in later textbooks (Black, 1936), including narrow horizontal grooves (A, B), disc-shaped and

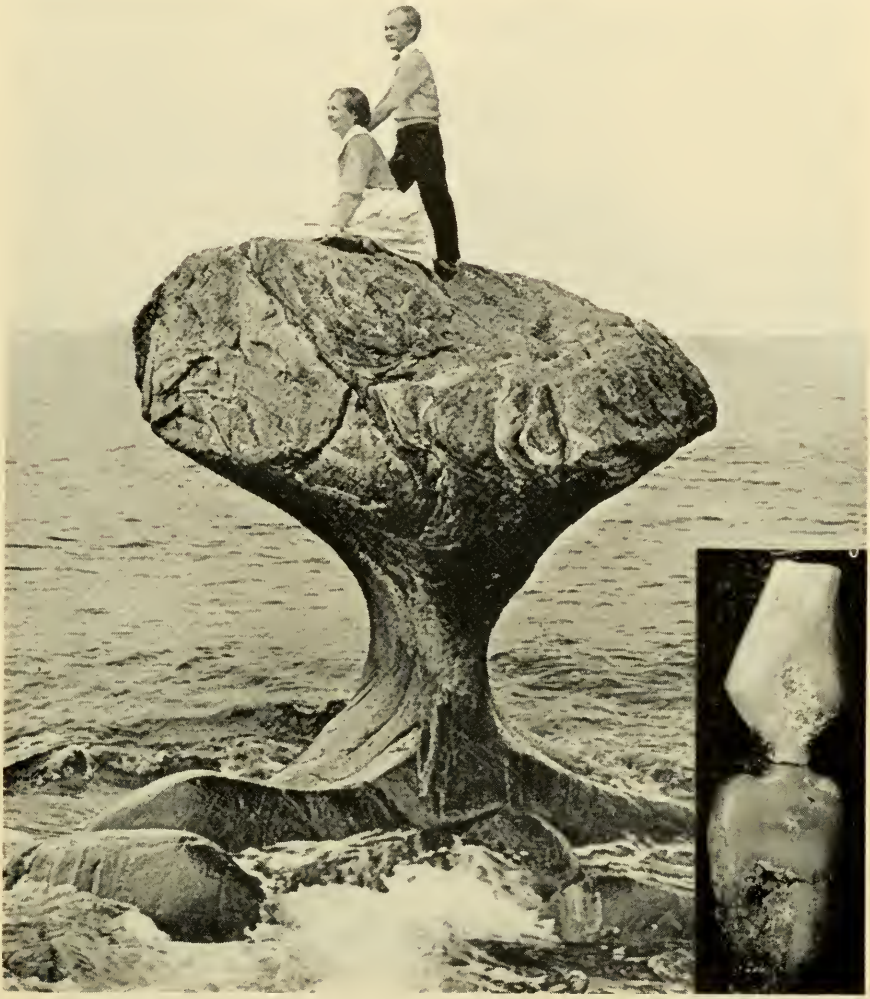


FIG. 2. Rock "erosion," curiously similar to certain circular erosions of human teeth (insert). (Reproduced from "The Toby-Jug Stone—Kannesteinen near Maaløy, Norway," with the permission of Mittet Foto A/S, Oslo, Norway.) In the insert is shown the ultimate "hourglass" appearance of one of the mandibular incisors seen in Fig. 1 I at an earlier stage of erosion. At the time of extraction, the erosion process had extended all around the lingual, labial, mesial, and distal surfaces.

flattened (C, D, E), irregular (F), figured (G), circumscribed, and wedge-shaped (H, I).

Though tooth brushing had been considered an etiological factor in erosion prior to the studies of Miller, it is noteworthy that several years before the turn of the century, Kirk (1887) emphasized that erosion could be found even in people who only brushed occasionally, sporadically, or not at all. Further, he noted, as we have amply illustrated above (Fig. 1), that there could be a great variation in the intraoral orientation of the lesions on different teeth. He found, furthermore, that people who brushed horizontally could nevertheless have lesions of erosion in the form of vertical grooves, that eroded teeth could be separated by non-eroded ones, and that erosion could occur, as we have noted, in teeth which were not accessible to the toothbrush at all.

Though it is true that typical dental erosion may ultimately assume a very wedge-shaped appearance with sharp angles, this is actually an advanced stage of pathology. In an earlier stage, we

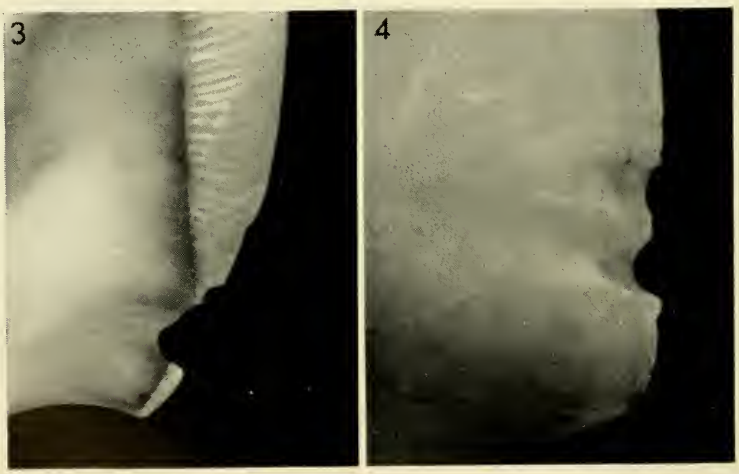


Fig. 3. Segment of labial surface of incisor with beginning erosion of enamel (upper part) passing through dentin-enamel junction at one point and, near cementum-enamel junction (below), producing deep wedge into the dentin. (× 12.)

Fig. 4. View of outer enamel surface removed from top of segment seen in Fig. 3. To the left of the erosion grooves, prior to gross loss, is seen a slight chalklike change in the opacity of the enamel. (× 12.)

have noted that the lesions, when observed grossly in tooth segments like those shown in Fig. 3, may have a multiple, rather rounded, "lacunar" profile, affecting similarly both enamel and dentin.

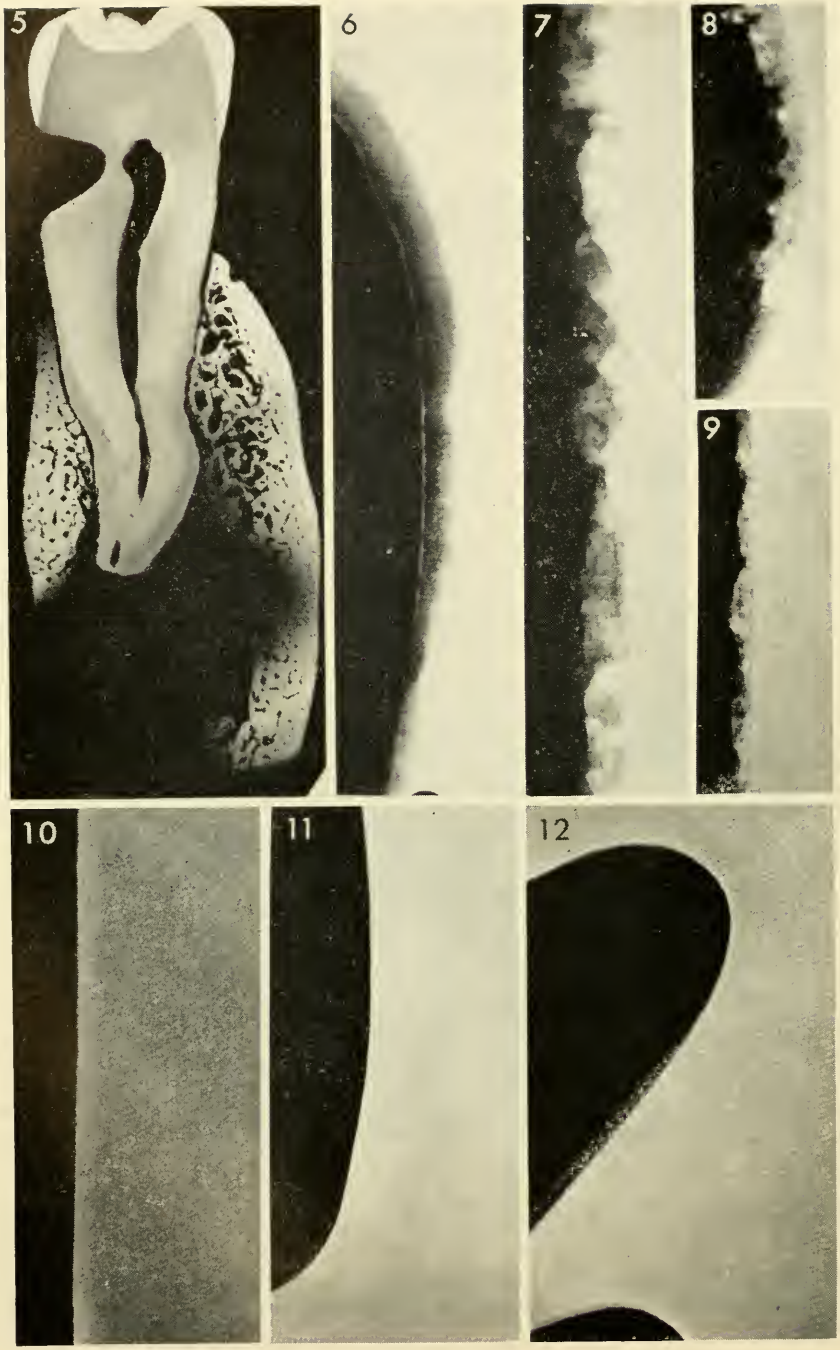
The peripheral surface of the beginning lesion in enamel is extremely hard to detect with the naked eye. With some magnification, however, as shown in Fig. 4, one may occasionally detect in the enamel surface, just outside the grossly visible defect, a very slight opacity or chalkiness. Clinically one would be unlikely to diagnose this important incipient stage, because to detect this, the tooth surface would have to be extremely clean, thoroughly dehydrated, and inspected with a lens under ideal lighting. We shall later discuss the structural basis for this appraisal. Suffice it to say, from the point of view of gross appearance, that if a beginning, posteruptive, chalky lesion in the enamel surface is seen readily with the naked eye prior to gross destruction, one can be reasonably sure that it is probably *not* erosion, but the beginning of caries (Sognnaes, 1940).

X-Ray Microscopy

It would seem a foregone conclusion that if dental erosion were caused purely by mechanical friction, then the surface of the lesion should exhibit a simultaneous and complete removal of all ingredients of the tooth substance, inorganic and organic.

Unfortunately, there is limited knowledge regarding the precise microscopic sequence of events in erosion. Textbooks on the pathology and histopathology of the teeth do not include any documentary evidence to indicate that the conclusions have been based on microscopic examinations. Indeed, it would appear that the characteristics of dental erosion mentioned, for example, in Kronfeld's *Histopathology of the Teeth* (re-edited by Boyle, 1955) are, in fact, based on the classical gross description of the lesions, stating that the lesions have "sharply outlined borders" and that the "floor is clean, hard and smooth in appearance."

This is not to say that information is readily obtained by routine histological examination. Neither ground sections nor decalcified sections examined in the optical microscope would in themselves



FIGURES 5 TO 12

necessarily reveal gradients in demineralization near the surface. Fortunately, however, through the recently refined method of microradiography, utilizing soft x-rays, between 5 and 30 kv, and a very fine-grained photographic emulsion (Kodak spectroscopic plates, 649-GH), it is now possible to produce very accurate x-rays of thin sections of bones and teeth which can be enlarged photographically under the microscope up to 1000 times. The accuracy of this method can be as good as 1 micron, making it possible to detect differences in microdensity and potential demineralization gradients near the surface of dental erosions.

Our own microradiographic observations have so far primarily been made on the superficial areas in lesions extending into the dentin, as exemplified in Fig. 5. In the bottom of these typical wedge-shaped lesions, we have been able to demonstrate that there can occur a change in the surface of the tooth substance, characterized by a gradient in the microdensity and indicating that demineralization may occur to a maximal depth of 100 microns (Fig. 6). This, it should be noted (as pointed out elsewhere: Sognnaes, 1959), is less than one-tenth of the demineralization gradient which can be observed in dental caries. In other words, in erosion the alterations are strictly confined to a surface effect. Occasionally the microstructure of the surface may exhibit a somewhat uneven configuration (Figs. 7 to 9), but even then there is no evidence of the deep destruction along the dentinal tubules so characteristic of caries. At other times, in certain parts of the lesion, the surface may be very smooth (Fig. 10), with little or no evidence

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Figs. 5 to 12. Microradiographs prepared by placing ground sections on fine-grained spectroscopic plates (Kodak 649-GH), exposed to soft x-rays (25 kv). In Fig. 5 the deep wedge-shaped buccal erosion cavity appears to exhibit no remarkable surface changes at low magnification (× 4). Figures 6 to 10 show at 100 times magnification that the erosion surface may exhibit a peripheral demineralization gradient. Figure 6, enlarged from bottom of wedge-shaped defect in Fig. 5, shows that this demineralization can reach a maximal depth of 100 microns. At certain stages the erosion lesions can be slightly irregular (Figs. 7 to 9), and at others quite smooth and dense as though there had been a redeposition of minerals in the periphery (Figs. 6 and 10). By comparison, mechanical abrasion, whether produced *in vivo* (Fig. 11) or *in vitro* (Fig. 12), shows very smooth lesions with no subsurface loss in microdensity.

of a detectable demineralization gradient. Indeed, the very periphery of certain parts of the lesion can at certain stages show a somewhat elevated microdensity (Fig. 6). It is quite possible that the lesion may assume a transitionally chronic stage in which there may be some slight redeposition of minerals in the organic matrix of the previously demineralized tooth substance.

For comparative purposes, we have examined lesions believed to represent uncomplicated abrasion *in vivo* (Fig. 11) as compared with destruction produced *in vitro* by mechanical friction (Fig. 12). For the latter purpose, extracted teeth were placed in a so-called tooth-brushing machine (courtesy of the Miami Valley Laboratories of the Procter & Gamble Company, Cincinnati, Ohio), and exposed to brushing with a tooth paste slurry for a period of time equivalent to years of tooth brushing under practical conditions. The surfaces of these lesions were similar *in vivo* and *in vitro* (Figs. 11 and 12), exhibiting a smooth contour without any evidence of subsurface demineralization. Admittedly a surface which has become demineralized, even though to a slight depth, could well be susceptible to further loss by mechanical friction, if within the accessible range of rigorous tooth brushing. In that case, this would be a secondary factor, assuming that our observation of a superficial demineralization gradient is indicative of some other chemical agent which, as a primary factor, would tend to demineralize the tooth surface.

Histopathology

Following the observation that dental erosion can exhibit a surface demineralization, at least at certain stages, it seemed of interest to explore more precisely, in stained decalcified sections, whether or not the lesion may be exhibiting any other characteristics which might explain this peculiar peripheral pattern.

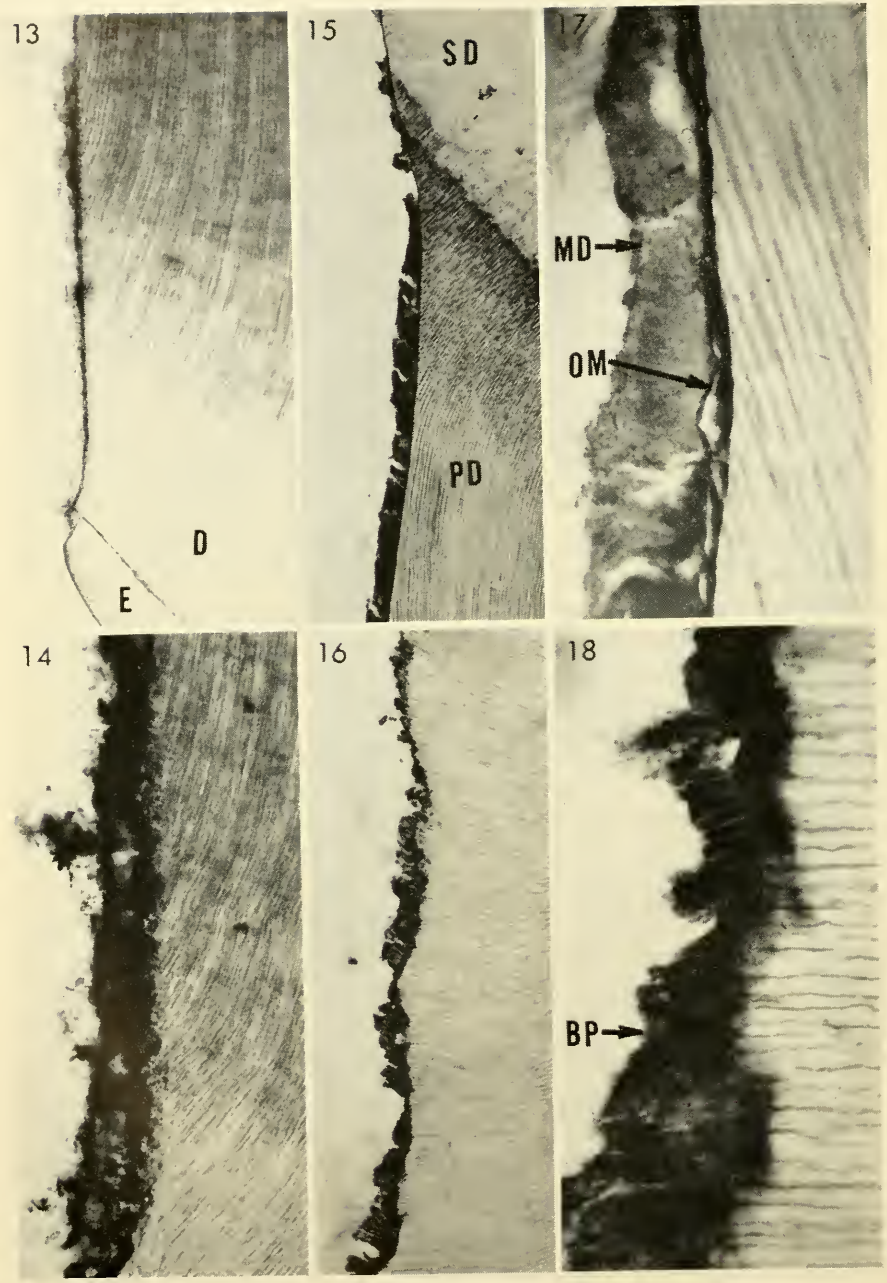
Paraffin sections were prepared following decalcification according to the method of Morse (1945). From a microscopic point of view, the active lesions did not always look as "clean" as would appear from gross appearance. Thus, we noted a thin zone of organic material attached to portions of the eroded surface, as illustrated in Figs. 13 to 18. In some instances (Figs. 13 to 15) the tooth surface was very straight and smooth, even though over-

lain by a thin mucous plaque containing microorganisms. In such cases we have noted a very thin cuticular membrane separating the microorganisms from the tooth surface proper.

At higher magnification (Fig. 17) this thin membrane may best be seen when stained with toluidine blue, with which it stains green, that is orthochromatically, in contrast to the bacteria-laden mucous film, which is intensely metachromatic with this dye. In other instances (Fig. 16) a metachromatic mucous plaque laden with bacteria appears to be in direct contact with the tooth surface without being separated by the orthochromatic organic film. Under this circumstance a superficial destruction of the tooth substance may be visualized. At higher magnification (Fig. 18) it is noteworthy that the surface destruction is primarily occurring in the matrix proper *between* the dentinal tubules. This is in sharp contrast to the bacterial invasion of the teeth in caries, in which the dentinal tubules become invaded at an early stage and to considerable depth prior to intertubular matrix destruction. Figure 18 illustrates this characteristic feature of erosion. The dark material represents bacteria situated in a mucous film, and it will be noted that the intertubular matrix is becoming destroyed without any widening of the dentinal tubules.

The possible bacterial implication in the etiology of dental erosion, suggested by these findings, does not appear to have received serious attention in the literature. This is perhaps not surprising in view of the fact that dental erosion usually looks so extremely clean to the naked eye. It must be remembered, however, that what is seen in the microscopic sections represents an extremely thin film, and since we have noted its relative absence in some areas of the lesions as compared with others, this film may be transitory and not always present.

The basic issue raised by these observations is whether or not the pathogenesis of erosion, albeit clearly different from that of caries (Sognnaes, 1959), is of inherently dental or oral environmental origin. Some peculiar characteristic of the tooth substance itself could theoretically invite destruction of the intertubular matrix along a surface area rather than invasion through the dentinal tubules in depth. On the other hand, the superficial environmental



FIGURES 13 TO 18

agent or agents, be they of bacterial, salivary, or other origin, could be such that their action would primarily attack the mineral phase in the intertubular calcified matrix rather than have the capacity to destroy and invade the tooth by way of its natural structural pathways. For the moment, this important question can be discussed only on the basis of very limited observations to be alluded to below.

Tooth Structure

The structural quality of the teeth themselves has received limited attention as a factor in susceptibility to dental erosion. Hopewell-Smith (1918) related a case of erosion which he believed was accentuated by faulty tooth structure, because sectioning of the eroded teeth also showed uncalcified interglobular dentin spaces. The latter defects, however, which have also been related to caries susceptibility, are exceedingly common as compared with erosion. Indeed, surveys on the microstructure of human teeth from various countries and periods of time indicate that such calcification defects are almost universally present in the teeth of man (Sognnaes, 1956).

FIGS. 13 TO 18. Tolnidine blue–stained sections through dental erosion lesions in the same mandibular incisor teeth seen grossly in Fig. 1 I at an intermediate stage, and in Fig. 2 (insert) at time of extraction for microscopic study. Figures 13 and 14 are plastic-embedded ground sections (× 100). The eroded tooth substance appears relatively smooth and clean near the outer enamel edge of the wedge-shaped erosion lesion (Fig. 13), which involves both enamel (*E*) and dentin (*D*). There is, however, a good deal of microscopic surface irregularity and organic debris in the bottom part of the erosion "cavity" (Fig. 14). Decalcified sections, Figs. 15 to 18, show variable amounts of dark-stained surface deposits. Figure 15 (× 100) shows a relatively smooth "cavity" surface and minimal debris even though the erosion has extended through the primary dentin (*PD*) into the secondary dentin (*SD*). Figure 16 (× 100) shows a microscopically more irregular erosion surface with varying amounts of organic debris. Figure 17 at higher magnification (× 700) shows a mucous metachromatic deposit (*MD*) about 15 to 20 microns thick separated from the smooth tooth surface by a delicate orthochromatic membrane (*OM*) 1 to 2 microns thick. This membrane may be protective since there is no significant active destruction of the underlying tooth substance. In Fig. 18 a zone of active tooth destruction has been enlarged (× 500). A bacterial plaque (*BP*) is here in direct contact with the tooth substance without any separating membrane. The intertubular dentin matrix is being destroyed, but unlike caries, there is no invasion of the dentinal tubules.

Were such calcification defects a primary factor, then erosion would be very widespread. At least it seems unlikely that faulty development can be blamed for the susceptibility to both erosion and caries. It is a general impression (Bodecker, 1953) that patients with dental erosion as a rule do not suffer from extensive acute dental caries, perhaps suggesting different underlying causes in the production of these two lesions.

It is interesting that two reports, by Bird (1931) and Kronfeld (1932), described independently the presence of abnormal excessive occlusal wear on the teeth among patients exhibiting dental erosion. Kronfeld even went so far as to relate the wearing facets to precise parts of the teeth, claiming that erosion tended to occur on that part of the tooth which was opposite to the side subject to abnormal wear. In other words, if the wearing facets were found toward the mesial edge of a tooth, the erosion would tend to occur toward the distal portion of the labial or buccal surface. One possible mechanism in this sequence of events was thought to be an effect of the traumatic occlusion on the pulp, the response of which then might indirectly affect the dentin structure. One could conceive of changes in the dentinal tubules resulting from changes in the pulp.

But in this chain reaction, one should perhaps still not overlook the possibility that the abnormal wear may have had an underlying relationship to abnormal salivary secretion. Undoubtedly the presence of an adequate flow and consistency of saliva could have a significant lubricating action during occlusal function. Extreme wear would be expected if completely dry tooth surfaces, unlubricated by saliva, were to grind against each other like dry rocks.

With respect to the indirect secondary effect of wear on the tooth substance itself by way of a pulpal response, the question has not been ruled out whether the pathogenesis of dental erosion may be related to some peculiar structure of the teeth themselves. We have noted above in our own microradiographic findings that the destruction accompanying erosion rarely penetrates along the dentinal tubules. These are never invaded to any depth by microorganisms as is found in caries. In other words, the possibility does exist that the tooth substance itself, owing to secondary obliteration of the dentinal tubules, may not give the same access to bacterial

invasion. At present this question is being pursued further at the ultrastructural level. Preliminary electron microscopic observations suggest that a peculiar form of large, dense crystals appears to be precipitated within the dentinal tubules in erosion (Frank and Sognnaes, unpublished).

Oral Environment

The above observations leave open the question as to the importance of some oral environmental effects, other than mechanical friction, as potential etiological factors in this peculiar type of idiopathic tooth erosion. There are few studies which have directly endeavored to determine whether agents potentially capable of demineralizing the tooth surface may be present in the oral environment in general, or in proximity to the lesion of dental erosion in particular.

Bodecker (1933) employed a simple clinical method of testing the environment next to the eroded lesions by means of wedges of litmus paper. These he inserted into the gingival sulci in juxtaposition to the eroded area, on the theoretical premise that there may be some acidic product of gingival inflammation which may contribute to the erosion. The latest tabulation of his findings (Bodecker, 1945) has been summarized in Table II. From his qualitative observations Bodecker inferred that the environment in juxtaposition to dental erosion more commonly tends to fall on the acidic side. In contrast, he concluded that the environment around normal teeth, especially in the younger age group, tends to be more on the alkaline side.

TABLE II. Simple Litmus Paper Determinations of Relative Acidity at the Sites of Dental Erosion versus Normal Control Teeth
(From data of Bodecker, 1945)

Condition of teeth	Age group	No. of patients	Percentage of distribution		
			Acid	Neutral	Alkaline
Sensitive erosions	21–61	42	67	14	19
Nonsensitive erosions	21–61	90	57	4	39
No erosions	21–61	151	16	25	59
No erosions	21–48	130	8	30	62
No erosions	23–24	56	5	5	90

The most significant single endeavor to test quantitatively and directly the general environment of the mouth in patients with erosion, as compared with controls, is a study by Zipkin and McClure (1949). They examined salivary citrate in 121 adult patients, 60 of whom had dental erosion. Statistical analysis of their data indicated a significant positive correlation between the presence of dental erosion and an elevated citrate content of the saliva. Both the citrate content of the saliva and the incidence and extent of erosion proved to be highest in the older age group, namely, above 40 years of age.

In apparent conflict with this observation is the study by Shulman and Robinson (1948), in which they found no difference in salivary citrate content between 15 freshman dental students who had dental erosion and 25 controls. This finding might appear to suggest that salivary citrate and erosion may increase independently of each other with increasing age, the two facts being coincidental rather than etiologically related. Young students, however, would be less likely to suffer from typical erosion, and the salivary citrate was increasing not merely with age, but with erosion and age. Theoretically citrate could serve as a calcium-sequestering agent (see chapter 25 by Jenkins and Dawes).

The possible implication of such a chelation influence in erosion is not new. As early as the 1880's it was postulated by Kirk (1887) that some agent in the mucous glands situated along the buccal and lip mucosa may be responsible for erosion. The idea was also expressed that this mucous secretion may be produced on a more continuous basis during the night—more so than the buffered secretion from the larger parotid glands. On the basis of some very limited observations, Badanes (1930) reported that oxalate tended to accumulate from the mucosa, especially on that side of the mouth opposite to the side upon which the person slept.

More recently there have appeared a few additional *in vivo* and *in vitro* observations with reference to certain calcium-binding agents. For example, Ericsson in Sweden (1953) examined the citrate level in the saliva after short periods up to 20 minutes after ingestion of orange juice and grapefruit. As might be expected, the citrate level rose sharply within the first couple of minutes, but

then dropped back to normal within 20 minutes. He found, however, that there was no excess secretion of citrate through the salivary glands when the citrate was given in a capsule swallowed prior to the test. In other words, it would appear that the transitory oral citrate increase following normal ingestion was due to retention of the citrate in the oral cavity, rather than to recirculation through saliva.

In several *in vitro* tests Ericsson added citrate to saliva and examined the demineralizing effect of this mixture on powdered enamel by examining the calcium phosphate content of the solution after 20 minutes. From these *in vitro* tests he concluded that the presence or absence of citrate had no significant effect, whereas acidifying the salivary mixture to pH 5.5—the presence of acid, in other words—did increase the enamel solubility.

Demineralization observed in rats following ingestion of solutions containing high amounts of citrate led McClure and Ruzicka (1946) to compare the effect of citrate and of lactate added to the drinking water and adjusted to a pH range between 5.5 and 7.2. When the molar teeth of the rats were weighed upon sacrifice after 2 weeks, relative loss of minerals was determined by the ultimate molar weight. These workers concluded that the acidity of the solutions was particularly destructive at the lower pH, as would be expected, but that citrate was significant in the sense that, like other chelating agents, it could be relatively destructive even at neutral pH and above.

Recently there has been considerable writing on the possible implication of a chelating mechanism in the production of erosion as well as of caries (Schatz *et al.*, 1957). In these studies, it was shown that oral proteolytic bacteria can dissolve pulverized rat bone and synthetic hydroxyapatite and that mixtures of salivary flora in various keratinous media have a high catabolic activity on cow and rat bone. The mineral withdrawal was attributed to some chelating effect of keratin or gelatin derivatives or cell metabolites acting as chelators. Unfortunately there is virtually no research on this problem directly related to dental tissues per se, let alone to caries or erosion. Though it may seem attractive to consider a chelation mechanism not requiring an acidic pH, it must be recalled

that Bodecker (1945) has hitherto made the only direct observations on the environment in juxtaposition to dental erosion. These findings led him to conclude that the erosion environment was distinctly on the acidic side, a condition not requiring the involvement of a chelating mechanism operating at the alkaline pH range. More precise tests are badly needed.

The assumption has been made that some keratinolytic microorganism or agent must be present as a primary factor in the destruction of enamel. On this theoretical ground, Schatz *et al.* (1957), again from indirect observation, have concluded that wool fibers may be proteolyzed by organisms indigenous to the oral environment. In the first place, however, the very classification of the organic matter of enamel as a keratin has been seriously questioned in newer studies which suggest that the enamel protein is not typical of either keratin or collagen (Piez and Likins, 1960; Glimcher *et al.*, 1961). Furthermore, we have recently been able to demonstrate that during mineralization of enamel, the fibrillar protein becomes so completely petrified that it would appear to be virtually inaccessible until the mineral component has been removed (Frank and Sognnaes, 1960; Frank, Sognnaes, and Kern, 1960). Until far more definitive facts are presented, one feels obliged to conclude, therefore, that some agents capable of removing the mineral fraction of the enamel would seem to be intimately and probably primarily involved in this type of hard tissue destruction, both in erosion and in caries (see also chapter 25 by Jenkins and Dawes).

Whatever the agent may be that is responsible for such a primary dissolution of the mineral phase of the teeth in the initiation of erosion, it seems obvious that a combination of such dissolving effects with mechanical friction would lead to greater destruction than one factor alone. This can be readily demonstrated *in vitro*. Indeed, W. D. Miller (1907) arranged teeth in an artificial toothbrushing machine and showed that the abrasive action of a combination of gritty paste and powder, following prior exposure of the teeth to acid, would result in greater tooth destruction than either one of these exposures alone. More recently Steel and Browne (1953) noted the more interesting point that teeth which, prior to

the application of acid solutions, had been thoroughly cleaned were much more susceptible to the acid action than teeth which were not first cleaned, but had retained some organic films on the surfaces.

General Constitution

In addition to the more obvious suspicion of oral environmental factors, there has appeared in the literature considerable speculation regarding other potential etiological influences on dental erosion. These range from general systemic conditions to local peculiarities within the mouth and teeth themselves.

A number of descriptive terms have been used to characterize the type of patient susceptible to erosion, such as the "neurasthenic" patient, the "live-wire-type," the "better class," etc. The late Dr. Bunting (cited by Hill, 1949) associated the development of these lesions with nervous strain and thought the condition became arrested with improvement in general health. Before the turn of the century dental erosion was related to a general systemic condition which was classified as "gouty diathesis" (Darby, 1892); the dental erosion patients were described as "neurasthenic" (Kirk, 1887). Others have seen a tendency to alcoholism among their dental erosion patients, but whether or not this might have been merely secondary to a more basic nervous condition may perhaps have been hard to tell in such cases. Yet it would be unwise to discard the potential significance of such long-standing clinical impressions. In an effort to bring the sequence of events within the framework of some logical relationship to the oral environment, one may perhaps consider four possible chain reactions that could bridge the dental and general state of health: (1) an indirect systemic effect on salivary secretion; (2) formation of oral debris of endogenous origin similar to what may be found in general fever conditions; (3) changes in the nature and habits of eating, drinking, and oral hygiene; (4) a relationship to masticatory function which could alter both the oral environment and the teeth themselves.

Clinical Management

At present the rationale for management of dental erosion can be discussed only on highly theoretical and tentative grounds. For this

disease is but one of many unfortunate examples of oral pathology where basic knowledge is sorely inadequate for intelligent integration with clinical practice. In particular, we lack the means of precise clinical measurements by which to diagnose early lesions and delineate, in each individual patient, the relative significance of the various structural, functional, environmental, and constitutional influences enumerated above. Only with such means for obtaining knowledge of the *individual* patient situation can one hope to arrive at a truly professional and scientific approach to diagnosis, prognosis, prevention, and therapy.

With this reservation, the following practical points may be made. Because erosion, unlike caries, fails to produce subsurface demineralization beyond 100 microns, one cannot diagnose the initial incipient surface lesion by the naked eye. The opacity, if any, is too superficial. Better diagnostic "eyes" are needed. The first clinical detection of a patient's tendency to erosion must therefore —in view of our present diagnostic inadequacies—await the manifestation of some grossly visible loss of tooth substance, that is, a very advanced stage of the lesion, biochemically speaking.

On theoretical grounds, our microradiographic findings (suggesting a primary surface demineralization) would lead to the conclusion that *one* preventive approach would be to reduce the solubility of the tooth mineral. To date there is no demonstrable practical way to achieve this better than the topical application of fluoride. Possibly a somewhat stronger concentration (4 per cent) utilizing the more soluble potassium fluoride (Sognnaes, 1941) and more frequent applications to the erosion-susceptible tooth surfaces (every 4 months) may prove advantageous. One may hope that other trace element combinations, such as the inclusion of heavy metal ions as exemplified by tin fluoride, may be brought to bear upon the clinical control of both erosion and caries. To my knowledge there have been no direct comparisons between the relative incidence of erosion and that of caries in fluoridated communities. This should be established. Even without fluoridation it has been an impression that a reverse relationship exists between these two types of oral pathology to begin with. But even though the oral environ-

mental factors may be different in the two diseases, the teeth themselves may be protected in both by decreasing the solubility of the tooth substance, irrespective of the type of dissolving agents.

Because erosion has a tendency to recur even after restoration of the gross defect, one may by the same token suggest that the tooth substance along the filling margins be similarly fortified by topical application of fluoride at regular intervals. With respect to the nature of restoration, certain silicates may have the advantage of their own fluoride contamination, which has been thought to be a factor in limiting recurrence of secondary caries along the filling margin. This possible advantage is, however, offset by other chemical and physical inadequacies, noticeably in the class V type of restoration often called for in the management of advanced wedge-shaped cervical erosion.

The observation was made above that the erosion lesion, despite its seemingly "clean" look to the naked eye, may be the site of a microscopic bacteria-laden mucous plaque, potentially involved as a destructive factor. This plaque, as well as other environmental contacts with lip, cheek, tongue, mucous glands, etc., would presumably prevail even after the eroded defect has been restored by a filling or inlay. This being so, it may conceivably be of advantage to so mold the restoration that the above chemical and physical influences are concentrated against the bulk of the restoration rather than the margins of the tooth substance.

This reasoning might lend support to a practical modification of cervical restorations brought to my attention through a personal communication by Dean Maynard Hine of Indiana University (original publication, if any, is not known). According to this procedure, the class V inlays for restoration of cervical erosion should be provided with a slight central concavity rather than convexity. In other words, instead of making the usual cervical contour (appropriate as this would be for protection of the gingival margin), the rationale would be to invite environmental agents to settle into some shallow depression in the center of the surface of the restoration. It is possible that this artificial duplication of a filling profile, which to a slight degree mimics the profile or contour of the early erosion

lesion itself, might confine the injurious influences to a relatively inert restoration material rather than to the adjacent tooth substance itself.

In view of the possible "eroding" influences of direct contact between the lip and cheek and certain erosion-prone labial and buccal tooth surfaces, it would be of interest to determine, furthermore, whether some thin separating paraffin paper or plastic shield, placed in the oral vestibulum during the night at least, might help to stagnate extreme cases of erosion.

In our effort to control this peculiar wasting of the teeth, several additional factors merit attention. Among these would be—as indicated by what has already been discussed above—elimination of occlusal trauma; evaluation and adjustment of chewing habits; analysis and professional advice regarding consumption of food and drinks; and, first and last, determination of, and when possible improvement in, the patient's general health and well-being.

Postmortem "Erosion"

In connection with an extensive study of the developmental quality and histopathology of ancient teeth (Sognnaes, 1956), it was discovered that various postmortem changes including erosions had occurred.

The investigation was based upon a study of human teeth which date back to eleven periods of history, paleolithic to recent, and originate from six geographic locations, Palestine, Egypt, Greece, Iceland, Norway, and Central America.

In terms of numbers of specimens from each source the material was limited, particularly the rare specimens of paleolithic Palestinian, predynastic Egyptian, and Old Icelandic teeth. The Greek material contained samples from the longest span of time within one and the same country, ranging from prehistoric to New Greek time. All these Greek specimens were obtained during the Unitarian Service Committee's Medical Mission to Greece in 1948, through the courtesy of Professor John Koumaris, Director of the Anthropological Museum of Athens.

The teeth from the Norwegian Middle Ages were obtained from the anthropological collections at the University of Oslo, through the late Dr. E. K. Schreiner, former Museum Director.

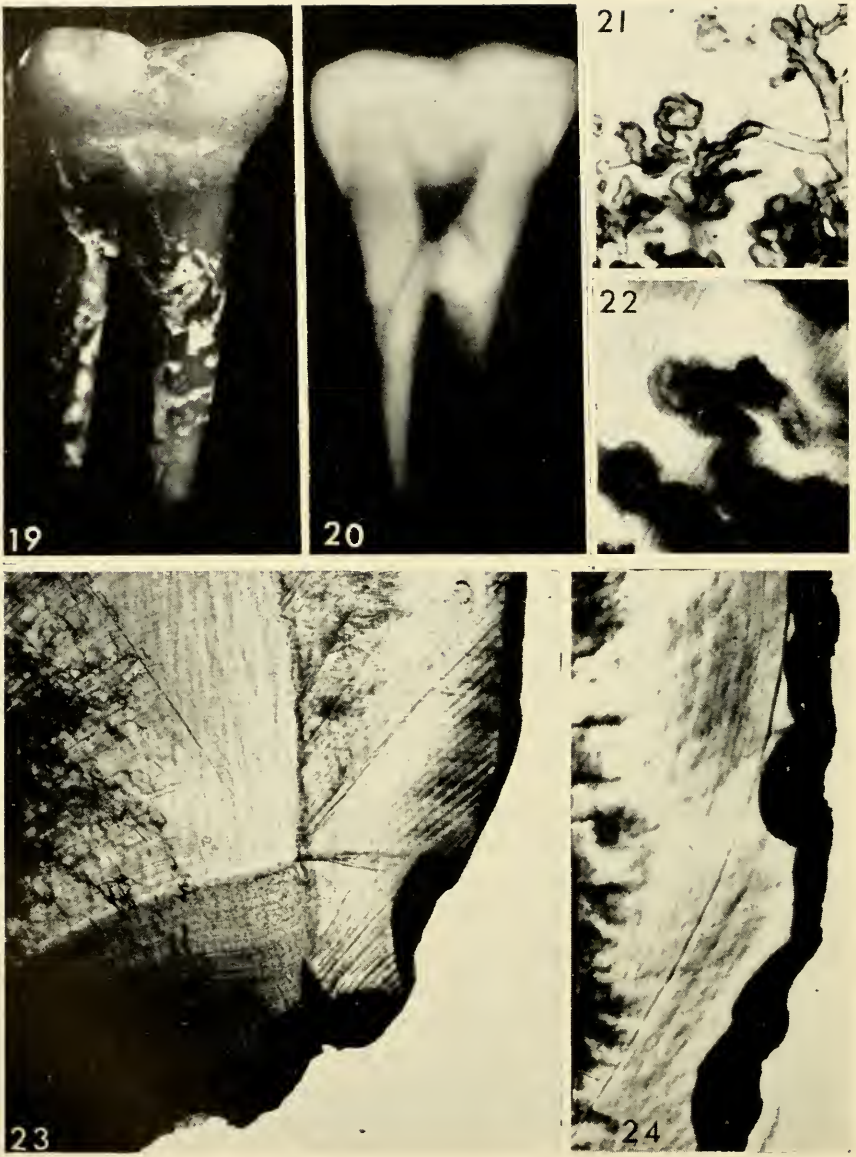
The Pecos Indian teeth, which represented the largest number from one single source, as well as the predynastic Egyptian and Guatemalan specimens, were all obtained from Harvard's Peabody Museum, through the cooperation of the late Professor Earnest A. Hooton, who gave much impetus to this investigation. Through his interest and that of Professor Hallan L. Movius, also of Harvard's Peabody Museum, it became possible to examine histologically several valuable specimens from the Stone Age people of Mount Carmel in Palestine, originating from the upper Pleistocene period.

Gross Examination

Examined grossly, before the teeth were prepared for histologic examination, the enamel covering the crowns of the teeth was generally surprisingly well preserved. Even some of the oldest specimens had retained a normal hard and glossy enamel surface. Occasionally, localized areas of the enamel surface exhibited a dull porous appearance as if etched by acid. Minor enamel fractures were of common occurrence. Thus, more than one-third of the teeth from various periods of Greek history presented grossly visible cracks and fractures, which were probably due to drying and handling.

The root surface of many of the exhumed teeth presented a dry, porous, and dull appearance. Upon further examination such specimens could be divided into two categories. In one, the x-ray absorption and weight of the teeth were equal to or exceeded the average for normal teeth. When submerged in 10 per cent nitric acid, the roots of such teeth seemed to be abnormally acid resistant. Removal of the mineral component left a meshwork unlike the leathery matrix of freshly extracted teeth similarly decalcified. This difference may have been due to a partial replacement of destroyed areas of dentin by highly mineralized matter.

The other category presented a similar surface appearance (Fig. 19), but the x-rays readily penetrated a large portion of the teeth (Fig. 20). The radiolucent area was most evident at the tip of the



FIGURES 19 TO 24

roots but also extended into the crown dentin, thereby giving the roentgenological appearance of undermined enamel. Furthermore, a narrow border of dentin around the wall of the pulp chamber often appeared to have become similarly radiolucent. The latter type of specimen weighed less than normal teeth of the same size and could be completely decalcified in a relatively short time, less than that required for freshly extracted teeth. Instead of having a leatherlike matrix like normal teeth, the decalcified roots could be pressed together like a sponge. This crude observation indicated that this type of tooth, besides the loss of the normal mineral component, had also suffered some loss of the organic framework. In further contrast to a normal dentin matrix, the remaining organic meshwork could easily be teased apart under a dissecting microscope and separated into shining bundles reminding one of the appearance of tendons. Through further examination it could be confirmed that this material had retained the pattern of collagenous fibers, in spite of the fact that the teeth had been buried in the ground for hundreds of years.

All in all, about 40 per cent of the Greek teeth, particularly the roots, exhibited the grossly visible porous appearance of the external surface. Whether this was due to simple acid erosion or to changes in the underlying tooth substance could not be determined by gross inspection.

FIG. 19. Molar tooth from prehistoric Greece, photographed by reflected light. The enamel surface has retained its normal glossy appearance, while the root has a dull, porous surface. (× 3.)

FIG. 20. X-ray photograph of the molar shown in Fig. 19. The white outline of the enamel cap indicates normal x-ray absorption, whereas the root, especially its apical region, shows unusual radiolucency, indicating loss of the normal mineral content. (× 3.)

FIGS. 21 AND 22. Photomicrographs of ground sections, showing different patterns of postmortem canals penetrating the dentin of teeth from prehistoric Greece (from Asine, Peloponnesos, Mycenae). For details see text. (× 90.)

FIG. 23. Ground section of mandibular right permanent second molar of adult female from the Skhul cave of Mount Carmel, middle paleolithic Palestine. In addition to postmortem canals (dark patches), there is a cervical "cavity" involving enamel and dentin erosion. (× 30.)

FIG. 24. Section of prehistoric Greek molar from Olynthus, Macedonia, buried for nearly 4000 years. Postmortem surface erosions of the enamel cut abruptly across the growth lines, without sign of vital reactions in the underlying tooth structure. Ground section, carmine stain. (× 30.)

Microscopic Examination

The procedure used for microscopic sectioning of the teeth was a petrification technique which utilized a plastic material, methyl methacrylate, for infiltration, hardening, and protection of the brittle specimens during sectioning and grinding (Sognnaes, 1947). In addition, a few smaller slabs from selected specimens were prepared as decalcified paraffin sections.

Histologically, it was possible to distinguish between several types of postmortem changes in the teeth. The enamel exhibited surprisingly few histologic differences in appearance from that of freshly extracted teeth. Cementum, dentin, and adherent alveolar bone, on the other hand, showed striking histologic alterations that could hardly have been predicted from gross inspection of the outer surface of the specimens.

Boring canals. It was found that abnormal canals, unlike anything observed in freshly extracted specimens, either entered the teeth from the inside through the predentin or penetrated the cementum-covered roots from the outside. More than one morphological pattern of these canals could be recognized. One type was characterized by long and narrow canals which often penetrated the whole width of the dentin in irregular fashion (Fig. 21). These canals were 2 to 10 microns wide, occasionally wider, sometimes exhibiting a corkscrewlike penetration, sometimes with several branches, 15 to 25 microns thick. This seemed to be the commonest type. The largest canals, and hence the most destructive ones, sometimes reached a diameter of from 50 to 100 microns and were easily identified by the ampulla-shaped widenings shown in Fig. 22.

Two of the most significant characteristics of these canals were, first, their great width, which exceeded that of the normally present dentinal tubules, and, second, the fact that the canals penetrated in all directions and across the intertubular matrix rather than along the dentinal tubules. Where the pulp cavity of the teeth was readily accessible through a wide apical foramen, the canals were most frequently found to enter the less calcified predentin zone and from there to proceed toward the periphery. This type of change was

particularly common in teeth which presented a dull, porous appearance of the external root surface, a lower than normal weight, and a greater penetration by x-rays. The presence of the canals in the dentin explains the enhanced transmission of x-rays as well as the scattering and, hence, hampered transmission of ordinary light through ground sections, especially when viewed at low power.

The cementum covering the root dentin of the teeth did not prove a barrier to the agents responsible for the canals. Where primary penetration of the cementum occurred near the enamel junction, the destruction might clinically and roentgenologically be mistaken for cementum caries. Histologically, on the other hand, there were distinctive differences between the postmortem penetration and the intra vitam type of invasion characteristic of caries. Unlike caries, which usually follows definite pathways in a consistent relationship to the morphological pattern of the teeth, the postmortem canals were very irregular, running across rather than along the direction of the dentinal tubules. Another histological characteristic, hinting at postmortem destruction, was the absence of vital response in the neighborhood of the "cavities" produced in the teeth after death. No evidence of either secondary or transparent dentin, both of which are familiar reactions to intra vitam lesions of the teeth, could be observed in a logical proximity to destruction of the type just described.

The enamel, which during life is so extremely susceptible to destruction in the form of dental decay, was not found to be invaded or destroyed by the postmortem canals described above. The enamel protein is very sparse and differs from typical collagen.

Diffuse disintegration. Occasionally, the teeth had lost their normal microscopic appearance owing to a more diffuse change in the dentin. Ground sections of such teeth had lost their normal transparency and appeared as if decalcified in strong acid, and yet the hardness of the tooth substance indicated the presence of a high content of relatively homogeneous inorganic matter believed to be caused by secondary petrification.

Surface erosion. In addition to the above-mentioned changes in the internal tooth substance, the surface of the teeth occasionally

had an etched appearance. When the teeth were stained *in toto* with carmine or similar dyes, such etched regions were easily penetrated by the stain. This condition was quite common on the cementum surface and was the only definite postmortem change found in the enamel. Occasionally this process had advanced to a point where it became grossly visible, the appearance being as if the surface had been etched. Histologically, these lesions were irregular, as shown in Figs. 23 and 24, unlike the diffuse etching produced by an even exposure of a tooth surface to acid. It is interesting to note in this connection that all the cementum and dentin may occasionally be entirely lost as a result of the postmortem canals described above, leaving complete shells of enamel as the only bodily remains in the burial ground.

Distribution of the Postmortem Changes

All together about two-thirds of the exhumed teeth exhibited one or more of the three aforementioned types of postmortem disintegration (Table III).

The penetrating canals proved to be the most frequently found type of destruction. In the Greek material about 60 per cent of the affected teeth were invaded from the external cementum surface ("external canals"), 70 per cent from the pulpal or internal dentin

TABLE III. Postmortem Erosions in Exhumed Human Teeth
(From data of Sognnaes, 1955)

Origin	% teeth with postmortem changes	% with erosion
Greece, prehistoric	80	30
Greece, 750–338 B.C.	70	25
Greece, 338 B.C.–1800 A.D.	80	0
Greece, 1800–1900 A.D.	25	4
Palestine, paleolithic	100	40
Egypt, predynastic	0	0
Iceland, ancient	0	0
Norway, Middle Ages	90	0
American Indians, Guatemala	60	0
American Indians, Pecos	70	0
All samples	70	10

surface ("internal canals"); in other words, many specimens were attacked from both inside and outside. This type of destruction consistently exceeded in frequency the other types in all the material studied, except the few teeth obtained from the Guatemala Indians, which showed predominantly a more diffuse disintegration.

Typical surface erosions were less frequent. In the Greek material, approximately 40 per cent of those teeth which were affected by postmortem changes showed histologically recognizable erosions on the cementum surface of the roots, and 30 per cent on the enamel surface of the crown. The degree of postmortem disintegration gives no reliable clue as to the age of the various groups of material. Of the 23 teeth from the Norwegian Middle Ages, less than 500 years old, nearly all (90 per cent) showed postmortem changes, exceeding the frequency found in the teeth from prehistoric Greece, which had been buried for several thousand years.

There was a suggestion, however, that the teeth obtained from the various periods of Greek history have become increasingly affected with the increase in postmortem age. Thus, 80 per cent of the prehistoric teeth were affected as compared with 25 per cent of those from the New Greek period. The erosions showed the most consistent increase in frequency with increasing postmortem age of the material. In fact, the only Greek teeth which exhibited this type of disintegration in a relatively large number of cases were those dating from the prehistoric period. The fact that no postmortem changes were observed in the teeth from the predynastic Egyptian and the Old Icelandic period may only serve to show that the postmortem age of single teeth in certain burial grounds cannot be predicted by the absence, presence, type, or degree of postmortem disintegration.

Origin of the Postmortem Lesions

Among the various dental diseases described by Hopewell-Smith (1918) in his textbook on histopathology of the teeth, he refers to two cases of a disease which he calls "fungoid excavations." The sections which Hopewell-Smith used for illustrations show what appear to be types of canals in the dentin similar to those which in the present study have been attributed to postmortem changes. He

suggests that these canals represent a new and extremely rare type of pathology occurring during life. In view of our present observations, however, it is significant to note that the two cases referred to by Hopewell-Smith actually were observed in exhumed teeth, in which we have seen that the condition, on the contrary, is extremely common.

Until similar canals are demonstrated in sections of freshly extracted teeth, it must, therefore, be concluded that we are dealing with a condition which has not been proved to occur during life but is very common after death. In further support of this contention is an accidental, but important and apparently little known, observation made about a century ago by Wedl (1865). He tells of having asked an employee at a morgue to extract and save some teeth before disposing of the dead. By mistake the teeth were left in a glass of polluted water for 10 days before being prepared for histologic examination. Upon sectioning, Wedl discovered a number of previously undescribed canals penetrating into the dentin and cementum from the outside surface. When Wedl's drawings recently came to my attention and were compared with the photographs of the present study, especially those in Figs. 21 and 22, the similarity became immediately apparent. This characteristic postmortem dental destruction, as well as the canals which Roux (1887), twenty years after Wedl's report, observed in exhumed bone, may therefore more appropriately be termed "Wedl's canals," as suggested in a historical note by Schaffer (1895).

The external canals observed by Wedl were obviously produced under somewhat unusual circumstances, immediately after death and in the course of less than 2 weeks. It is not known whether the canals observed in our exhumed specimens were similarly produced shortly after burial, before the process of putrefaction set in, or later. Some of our observations suggest that much of the postmortem destruction must have taken place a longer time after burial. In the first place, it was noted that the internal canals occurred with greatest frequency. This means that the pulp, as well as the periodontal membrane and other soft structures, must first have undergone proteolysis and that many of the teeth must have been severed from the jaw before becoming accessible to the agents responsible for the

canals. Second, it has been noted that the regions bordering on cracks in the teeth are favorable sites for the invaders. Such cracks are most likely to have occurred some time after burial, owing to a drying-out process. Another favored site for the spreading of the canals is the junction between cementum and dentin and also the dentin near the dentoenamel junction. Here, again, the drying-out process would cause tension owing to the difference in coefficient of expansion between the two structures. This drying-out process would be expected to take some time, a fact which is in keeping with the greater frequency of the postmortem canals in the older teeth obtained from one and the same geographic region.

The agents responsible for the pattern of the postmortem destruction of the dentin and cementum, as observed at the microscopic level, have not been definitely established. A few specimens appeared to contain highly refractile thread forms within some of the canals, but these may have been artifacts caused by the drying-out process of larger organisms. Many of the canals were very similar to those thought to be caused by the invasion of fungi, as suggested by the early observations of Wedl, whereas Werner (1937) suspected algae and worms as being capable of causing similar canals. The fact that the canals vary in width and extent of penetration would, however, not necessarily mean that different types of organisms are involved, because their pattern may well depend upon the conditions met along the way, which may allow for periodic lateral action instead of forward penetration. The secondary infiltration of minerals noted in some of our dental specimens may be similar to that observed in old exhumed bone and attributed largely to deposition of calcium carbonate.

Various agents may be responsible for the surface destruction which was observed in the cementum and which represented the only significant postmortem change in the enamel. The fact that the enamel, which is the most exposed and the most acid-soluble part of the teeth, was usually less affected than the cementum suggests that the erosions are not due to a generally acid environment entirely surrounding the jaws and teeth, but must be due to destructive agents localized at certain regions of the teeth.

These erosions do not have the appearance of caries except when

occurring at the neck of the teeth in combination with other postmortem changes. Investigators have in the past expressed doubts as to the intra vitam nature of caries-like cavities observed in the enamel of prehistoric teeth. It would seem from our observations that cavities in the enamel proper observed by gross inspection of prehistoric and ancient teeth may with some confidence be attributed to intra vitam pathology, hypoplasia, or caries, and, when doubts exist, the differential diagnosis may be established by histologic examination, as indicated above and elsewhere.

Acid soil would be expected to give a generalized surface etching all over the tooth, but this is rarely seen. The demarcated decalcification zones in the tooth surface are suggestive of a localized cause. In the case of the external tooth surfaces it seems logical to assume that the postmortem destruction would be favored by the aerobic conditions in the upper layer of the soil within a relatively short period after death, before bacterial and gaseous products of putrefaction become predominant. This does not, however, satisfactorily explain the location of the destruction in the internal parts of the teeth. The production of these defects would require longer periods.

Finally, one should perhaps not overlook the possibility that the defects described above may occasionally have been produced after exhumation of the skulls and teeth, in cases where the specimens, when stored, were covered with wet ground, in which case conditions would prevail similar to those reported by Wedl. For the major defects, however, this would seem to be a somewhat remote possibility, because the postmortem destruction has often been grossly evident at the time of exhumation, sometimes to an extent where only the caps of enamel remained. Such destruction is obviously not produced during life, but must occur sometime after death and before exhumation.

Experimental Demineralization and Remineralization

In view of the fact that the dissolution of calcium phosphate looms so significantly in the mechanisms of vertebrate hard tissue destruction, a few experimental observations (largely unpublished and

preliminary in nature) seem pertinent with regard to (1) *in vitro* and *in vivo* tests of the gross and microradiographic appearance of hard tissues exposed to demineralizing agents, and (2) *in vivo* capacity for remineralization of such partially demineralized lesions, followed by (3) a few comments on the theoretical and practical significance of such observations.

In Vitro Demineralization

In following up earlier studies on various demineralization gradients found in pathological destruction of enamel, dentin, and bone (Sognnaes, 1959), it appeared of interest to determine to what extent these gradients were due to structural characteristics of the tissues themselves.

With this in mind various hard tissues such as enamel, dentin, ivory, bone, and shells have been exposed to comparable chemical agents capable of withdrawing minerals. Furthermore, these agents have been placed in juxtaposition to the hard tissues with or without prior removal of the natural surface layers and with or without admixture of other agents that may influence the surface chemistry, such as organic polymers (hydroxyethyl cellulose) simulating salivary mucin. At this point, only a few observations will be cited, based on gross appearance and high-resolution microradiography (Fig. 25A to E).

When different mammalian hard tissues are exposed to identical environments capable of withdrawing minerals, only the enamel exhibits to any appreciable degree the characteristic opacity that is so familiar in the naked-eye appearance of incipient caries. The dentin and other mesenchymal hard tissues, even if exposed within the same over-all specimen to the identical agents, fail to exhibit these optical surface changes. This difference in appearance between enamel and dentin (Fig. 25C) in no way provides a true clue to what is going on within the tissue. In both cases there is indeed subsurface demineralization (Fig. 25D and E). Similarly one may conclude that the clean and intact-looking dentin surface in the bottom of a dental erosion lesion may not provide a true clue as to subsurface changes.

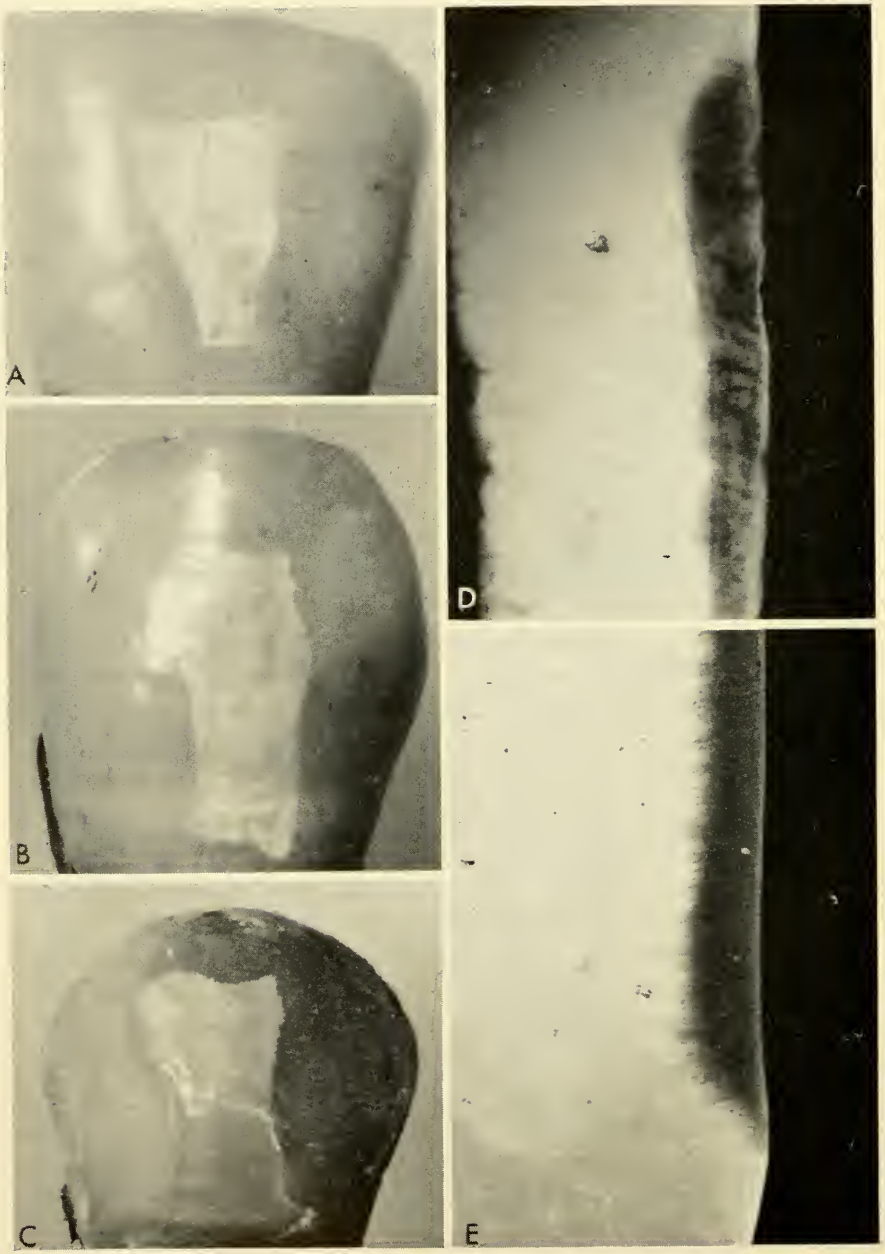


FIGURE 25

Irrespective of the type of hard tissue involved, the surface versus subsurface attack depends on whether the tissue is exposed to a simple demineralizing solution or to a mixture also containing an organic polymer. In the former case the dissolution is most intense superficially; in the latter case, there is present a thin relatively intact surface layer with considerable subsurface demineralization.

The typical pattern of incipient enamel caries—in which there is initially a relatively intact enamel surface overlying a considerably demineralized subsurface zone—can be duplicated *in vitro* by exposing untouched extracted teeth to a pure and simple decalcifying solution. In part, this may be due to the higher fluoride content and lower carbonate content of the outer enamel surface. But, in part, it appears to be caused by organic, natural surface coatings that influence the physical chemistry of the enamel, and in part by redeposition of some other mineral phase with different solubility properties (Sognnaes, 1962; Gray and Francis, this volume, chapter 8).

Even if the enamel surface is removed, the remaining enamel will exhibit the typical opacity and the relatively intact surface layer as well as the subsurface demineralization zone, provided the specimen is exposed to a demineralization solution containing an organic polymer simulating the mucoid of saliva (Fig. 25B and C). In other words, this pattern can be reproduced irrespective of the presence of the high-fluoride layer in the intact enamel surface. Indeed, this same subsurface demineralization (even though not visible to the naked eye) can be produced in relation to other hard tissue surfaces such as dentin, provided it is exposed to the polymer-containing demineralizing solution (Fig. 25E).

FIG. 25. *In vitro* demineralization of human teeth exposed for 96 hours to a buffered reagent (0.04 M lactic acid, 0.03 M $CaCl_2$ adjusted to pH 4.0 with NaOH) containing an organic polymer (6 per cent hydroxyethyl cellulose). Typical opacities have developed (A, B, C, × 10) whether the exposed white enamel "window" was produced on an originally intact tooth surface (A), on a pumiced subsurface (B), or on a deep internal layer of enamel (C, top), whereas no opacity is seen in the exposed dentin surface (bottom of C). However, in microradiographs (× 100) prepared from specimen C it is noted that a subsurface leaching out of minerals has occurred, both in enamel (D) and in dentin (E). Also note (D and E at right) the thin (white) zone of relatively less demineralized surfaces.

In Vivo Demineralization

In our initial experiments it was decided to produce enamel changes sufficiently extreme to be seen with the naked eye and recorded photographically in the living animal. In the rhesus monkey this was readily accomplished by exposing the enamel surface to a 5 per cent solution of nitric acid for 3 to 5 minutes. When the surface was washed with a stream of water and then dried by compressed air, one could readily detect areas of demineralization by the chalky, opaque appearance of the enamel.

Figure 26A illustrates the upper and lower incisors of a rhesus monkey before application of the acid. Figure 26B shows the appearance of the teeth 5 minutes after application of the acid. In the upper jaw, the central incisors were isolated with a rubber dam and acid was applied over the whole labial surface (Fig. 26B, top). In the lower jaw, a strip of gauze, soaked in the acid, was placed along the lower half of the labial surface (Fig. 26B, bottom). Here the chalky areas were quite clearly demarcated from the glossy appearance of the rest of the enamel surface. In Fig. 26C the same teeth are photographed 7½ months after application of the acid. Even though the teeth were dried thoroughly there was no appearance of any chalkiness of the enamel surface.

It next became necessary to find means whereby one could determine more precisely to what extent the enamel had been altered during various intervals of time following the acid demineralization. Thin ground sections of the teeth were prepared as described elsewhere (Sognnaes, 1947) and microradiographs were prepared at various intervals of time following experimental demineralization of the enamel surface of the teeth. The microradiographic films indicated that this method reveals considerable demineralization in depth. Furthermore, it was noted that the demineralization was not uniform along a broad front, but was permeating particularly along the lines of Retzius. This pattern was marked enough to be seen also in the sections of the same teeth examined in transmitted light. In addition, the demineralized areas took up a greater amount of stain than did normal enamel when immersed in toluidine blue.

The above tests made it evident that subsurface alterations in the

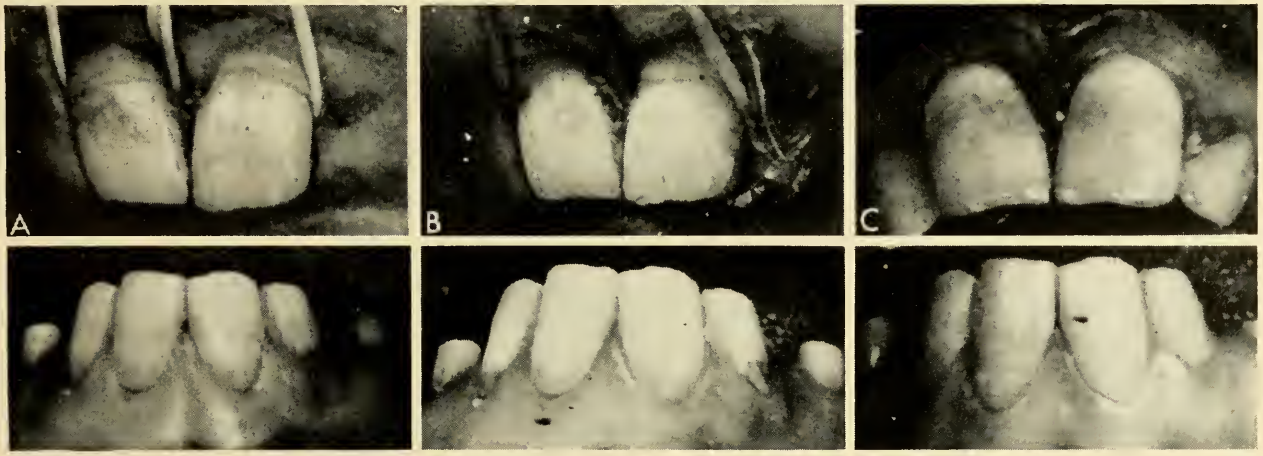


FIG. 26. Incisors of young rhesus monkey: A, before experimental demineralization; B, 5 minutes after topical application of 5 per cent nitric acid; C, 7½ months later. Note the changes in the opacity of the enamel surfaces. For details, see text. (× 4.)

enamel, following topical application of the acid, were greater than could be predicted from inspection and replicas of the enamel surface.

The microradiographic technique was, therefore, applied to teeth which had been etched with 5 per cent nitric acid and then left unattended in the mouth of these normal animals for various lengths of time. The results of the longest experiment done to date are shown in Fig. 27A, B, and C. The first illustration (Fig. 27A) indicates the depth of demineralization immediately following the application of acid. Seven and a half months later, when comparable teeth were extracted, the chalkiness was no longer apparent. Furthermore, upon sectioning of these teeth, it was noted that the demineralized zones along the Retzius lines seen in the microradiographs of control teeth had disappeared. Although an occasional defect still remained in the surface (Fig. 27B), there was actually a somewhat increased radiodensity in the superficial layers of the enamel (Fig. 27B and C).

Next it became of interest to determine the rapidity of these changes. In other animals, teeth were, therefore, extracted 2 days to a few weeks following demineralization. Grossly, we already observed differences 2 days after application of the acid. The corresponding microscopic appearance of the enamel is shown in Fig. 27D in transmitted light, and the microradiographic appearance in Fig. 27E, 2 days after application of the acid. Already it appears that the microdensity is somewhat increased. The enamel of teeth remaining in the mouth for 1 week after application of the acid is shown in Fig. 27F. At this point, there is a further increase in the microdensity of the enamel surface and disappearance of the previously pronounced Retzius lines. In these and similar tests in other animals, the additional observation was made that the enamel, even though it had regained an elevated microdensity, still manifested a greater stainability than intact enamel of the control teeth.

In view of the clinical use of orthophosphoric acid in the dental cement employed under orthodontic bands, it was considered of interest to observe the behavior of the enamel following application of this acid. Again, as shown in Fig. 27G, this acid also had a tendency to produce subsurface changes along the Retzius lines, even though the gross appearance of the enamel after short applications did not

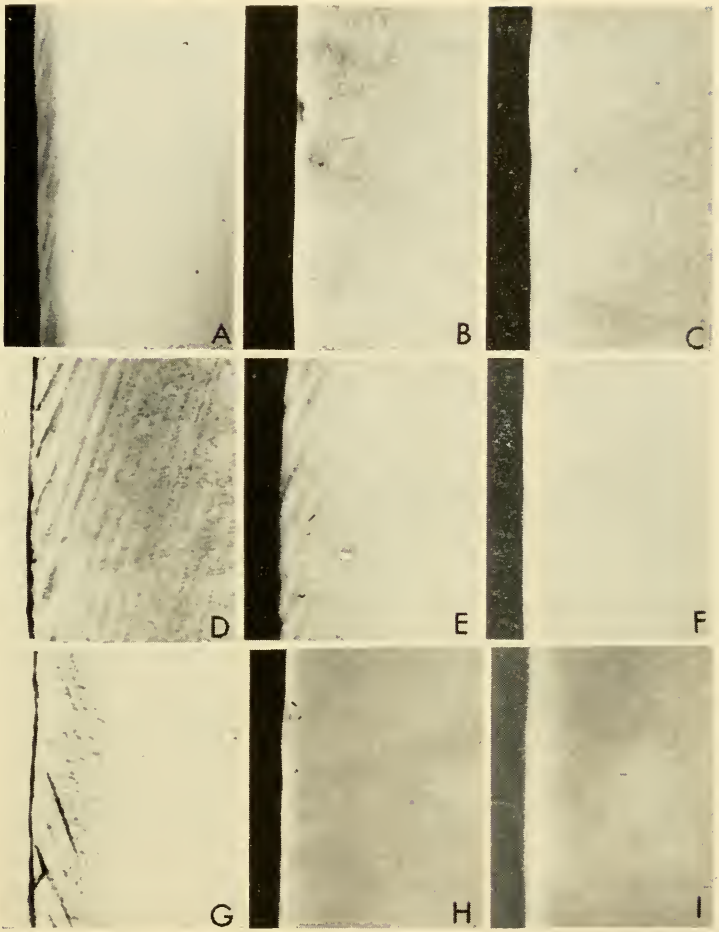


FIG. 27. A, B, C. Microradiographs of monkey incisors: A, immediately after etching with 5 per cent nitric acid for 5 minutes; B, C, comparable teeth examined 7½ months later. Note the apparent hypercalcification in the peripheral layer after "remineralization." (× 90.)

D, E, F. D, E, ground sections of enamel seen in transmitted light (D) and in microradiograph (E) 2 days after *in vivo* etching. F, microradiograph of comparable tooth 1 week after etching. Note dense surface. (× 90.)

G, H; I. G, etching with orthophosphoric acid (as used in dental cements) is seen to penetrate along the Retzius lines in the ground section shown in transmitted light. H, I, micrographs of comparable teeth, extracted 1 and 2 weeks later, show a resumption of microdensity in the peripheral enamel layer. (× 90.)

seem materially altered. When, however, the etched teeth were left in the mouth of the animals for 1 week after application of this acid, the same increase in radiodensity could be demonstrated in the enamel surface (Fig. 27H and I). In some areas, as shown in Fig. 27 I, this increased density did, in fact, exhibit itself to quite an extent below the external enamel surface.

The teeth of monkeys as compared with those of other animals offer the advantage of greater similarity to human teeth. But it seemed of interest to explore the usefulness of smaller rodents for similar demineralization tests. Though the relatively small molar teeth of rats do not lend themselves to isolated demineralization of discrete areas within single teeth, it proved possible to expose jaw quadrants of three molars to various agents, using other jaw quadrants for contrast. Such a scheme is illustrated in Fig. 28A to D.

After preliminary drying, the mandibular rat molars in quadrants of three were exposed to a 5 per cent solution of HNO_3 for a few minutes *in vitro* (Fig. 28A) and *in vivo* (Fig. 28B) respectively, littermates being compared at the outset of the experiment. In living littermate animals, the etched molars resumed nearly normal glossy surfaces in 6 hours after the etching (Fig. 28C), more fully normal 33 hours after the etching (Fig. 28D). In each case, the maxillary quadrants with the three untreated molars served as controls (Fig. 28A to D, top). Thus, from a clinical and esthetic point of view it would appear that the teeth of normal animals can tolerate an incipient surface etching and regain the seemingly normal glossiness of a healthy tooth surface.

It should not be assumed, however, that the partially demineralized tooth substance can become completely reconstructed to its precise original physical and chemical state.

In the first place, there is not necessarily a direct correlation between physical and chemical properties of teeth. The hardness and solubility of identical areas of ground surfaces of enamel on a large number of teeth were measured by Swartz and Phillips (1952). The enamel varied widely in hardness and solubility from tooth to tooth as well as from area to area on the same tooth. No consistent correlation between hardness and solubility was detected. The Ca/P ratio of the enamel dissolved during the solubility tests was slightly higher

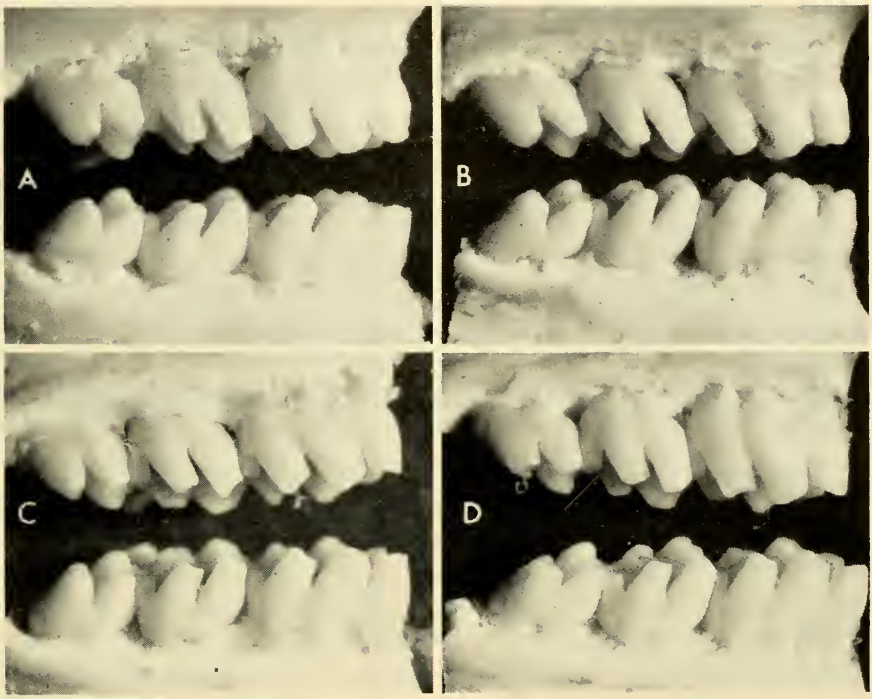


FIG. 28. Mandibular rat molars (in quadrants of three) were exposed, after preliminary drying, to a 5 per cent solution of HNO_3 for a few minutes *in vitro* (A) and *in vivo* (B). In the living animals, the molars partly resumed normal glossy surfaces 6 hours after the etching (C), more fully 33 hours after the etching (D). Quadrants of three untreated maxillary molars (A to D, top) served as controls. For details, see text. (× 7.)

than the 2:1 known to exist in whole enamel. Possibly the reason for this (in this writer's view) may be the presence of different proportions of phosphate and carbonate within the interprismatic regions, which are late to calcify, and which appear to be primarily involved in early subsurface demineralization.

Secondly, in our own experiments it was found in several of the "remineralized" specimens that whereas the microdensity appeared to be recovered within a week after application of the acid, there nevertheless appeared to be a greater microscopic dye-binding capacity within these areas of "rehardened" enamel. Moreover, in transmitted light (without any stain) the pronounced Retzius lines were

visible despite the fact that the radiodensity appeared normal. Even several weeks after the etching we could detect dye penetration in depth along the Retzius lines when sections were stained with toluidine blue. In this respect, the rehardened enamel (notwithstanding its high x-ray density) behaved differently from intact enamel of mature teeth. Possibly the affinity for toluidine blue suggests the presence of an amorphous mucous substance which could be contributed by saliva. The dye-binding capacity of the organic matter present in the rehardened enamel remained greater than in intact enamel, suggesting that we are not dealing with a true reconstitution to the original status of normal mature enamel, despite the seemingly dense deposition of inorganic matter. In other words, the normal organic-inorganic linkage of enamel (which has few if any free organic radicals) does not appear to become reconstituted. It would seem very inappropriate, therefore, to refer to enamel remineralization as a genuine healing process ("echte Heilungsvorgang," Rheinwald and Staehle, 1949), least of all a process attributable to a biological capacity inherent within the enamel itself as apparently implied in some earlier writings.

Theoretical Basis for Remineralization

On the strength of these experimental observations it would appear that the hard tissue destructions that affect the teeth are not entirely one-way processes, even within the seemingly inert biological makeup of the dental enamel, which is both acellular and avascular and in fact the densest of all biological tissues.

It is now more than three decades since Andresen (1926) presented the first comprehensive discussion of the potentialities for remineralization of enamel. In retrospect, it is noteworthy that his clinical interest in this subject preceded by ten years the theoretical concept of an ionic exchange mechanism in the seemingly stable dental hard tissues (this new biological principle did not come to light until Chievitz and Hevesy in 1936 for the first time traced the pathway of intravenously injected man-made radioactive phosphorus into the calcified substance of bones and teeth). Only recently has this concept been extended to definitive zones of enamel of adult

teeth of the primate species, as reviewed elsewhere (Sognnaes, 1955, 1957*a*, 1957*b*, 1960*b*).

Precise quantitative *in vivo* tracer studies in monkeys, utilizing high levels of various radioactive isotopes, have conclusively demonstrated the capacity of the enamel to take up radioactive elements from the salivary circulation and to a lesser degree from the internal connective tissue fluid of the pulp, depending upon the stage of dental calcification and eruption (Sognnaes *et al.*, 1955).

Of particular pertinence to the present discussion is the information obtained by studies on phosphate deposition in enamel as illustrated in Fig. 29. The arrows in this x-ray of the skull of a young rhesus monkey point to the relative degree of radioactive phosphorus uptake (8 days after injection of 5 millicuries of P^{32}) in the enamel of two unerupted and two erupted teeth (the corner figures, per cent of dosage $\times 10^7$ per mg of enamel, give three values, namely for surface, subsurface, and internal enamel, reading from top to bottom). It will be noted that of the two unerupted teeth, the second permanent molar (upper right) is slightly farther advanced in calcification (i.e. less "radioactive") than the first permanent premolar (upper left). The slightly younger tooth shows a radioactive phosphorus uptake per milligram of enamel more than twofold higher in the peripheral layer, more than threefold higher in the subsurface and internal layers. The two erupted teeth, which were exposed to the salivary environment, show that the central permanent incisor (lower left arrow), which had been exposed to the salivary mouth environment for a slightly shorter time than the first permanent molar (lower right arrow), has twice as high a radioactive phosphorus uptake in the peripheral enamel layers as does the "older" molar enamel.

In view of the fact that the two unerupted teeth were surrounded by the same internal environment (connective tissue fluid), it can be concluded that their marked difference in radioactivity must be due to considerable differences in the enamel structure itself. Presumably the permeability of the younger tooth enamel is greater, and the crystals are still relatively smaller and hence exhibit a relatively larger total surface for exchange as well as active growth of the inorganic apatite crystals.

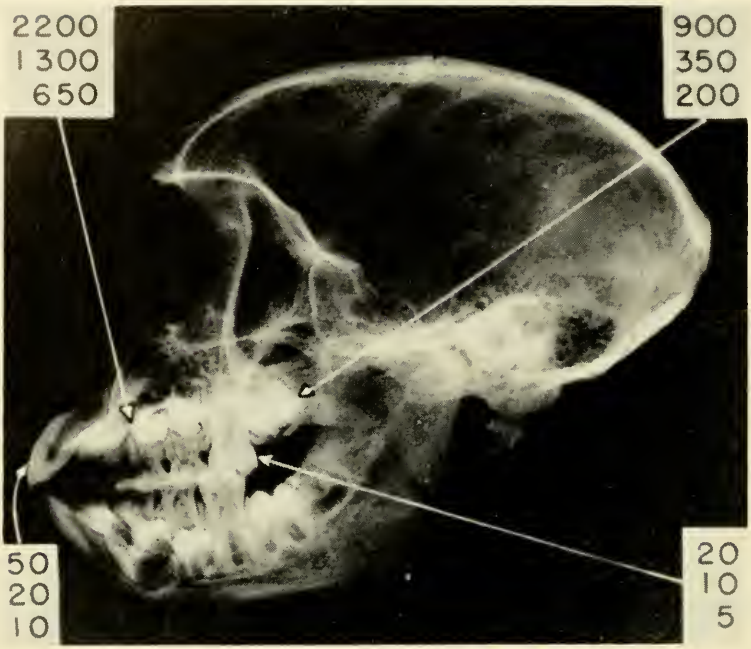


FIG. 29. Quantitatively determined radioisotope gradients within the outer layers of enamel of unerupted (upper arrows) and erupted (lower arrows) rhesus monkey teeth 8 days after intravenous injection of 5 millicuries P^{32}. Corner figures, reading from top to bottom, give values for surface, subsurface, and interior enamel in percentages of dose $\times 10^7$ mg of enamel. In each group of teeth, whether erupted or not, one notes that there is at least a twofold elevation of radioisotope uptake in the relatively younger anterior teeth (left arrows), indicating a prolonged mineralization period of the outer enamel, even after crowns are completed as far as can be judged by clinical x-ray diagnosis. (From Sognnaes, 1957*a*.)

By the same token, the two erupted teeth (lower arrows) were both surrounded by the biologically radioactivated saliva. Thus—and for the same reason as above—their different response must be due to differences not in the environmental reagents but in the submicroscopic structure of the enamel itself. It must be assumed that the most recently erupted incisor (which shows twice as high a radioactive phosphorus uptake as the first permanent molar) must still be in the stage of submicroscopic mineralization. In other words, some of the inorganic enamel crystals have yet to reach their full

size. Furthermore, there is evidence from related experiments that this final submicroscopic maturation of the enamel is to a large extent controlled by the liquid-solid equilibrium between the salivary environment and the enamel structure (Sognnaes, 1955, 1957*a*).

On the strength of these observations, the following reasoning suggests itself. If the enamel of erupting teeth, when confronted with a normal salivary environment, is capable of reaching a higher degree of submicroscopic mineralization of its crystals, the question arises whether fully mature enamel which has been reversed to a less completely mineralized stage by superficial pathological demineralization is then capable of reassuming the original, supercalcified stage, through contact with a favorable salivary environment. In other words (referring again to Fig. 29), one may ask whether the physiological mechanism which can bring the enamel mineralization from the stage of the recently erupted incisor up to the more highly mineralized stage represented by the molar enamel (Fig. 29, lower right) may be duplicated if the enamel of fully calcified teeth is experimentally or pathologically drained of some of its lime salt content. It is on the basis of this reasoning that the preliminary remineralization experiments described above were carried out and included in this report.

In this writer's view, it would seem that one can now approach this remineralization problem with less skepticism and better scientific tools than might have been the case only a few decades ago. For there is emerging both theoretical and experimental support for the hypothesis that incipient biochemical destruction of the enamel—even though it extends along certain susceptible pathways below the external enamel surface—has potentialities for reversibility within certain critical limits, yet to be fully explored in theory and exploited in practice.

From a chemical point of view it is interesting to note that Andresen (1926)—prior to these theoretical concepts—proposed a remineralizing salt mixture for topical application to partially demineralized enamel surfaces. His prescription was conceived essentially as a facsimile of the inorganic ingredients of saliva. Further developments may well be based upon a more selective composition and hence a purer chemical reaction with enamel with due regard

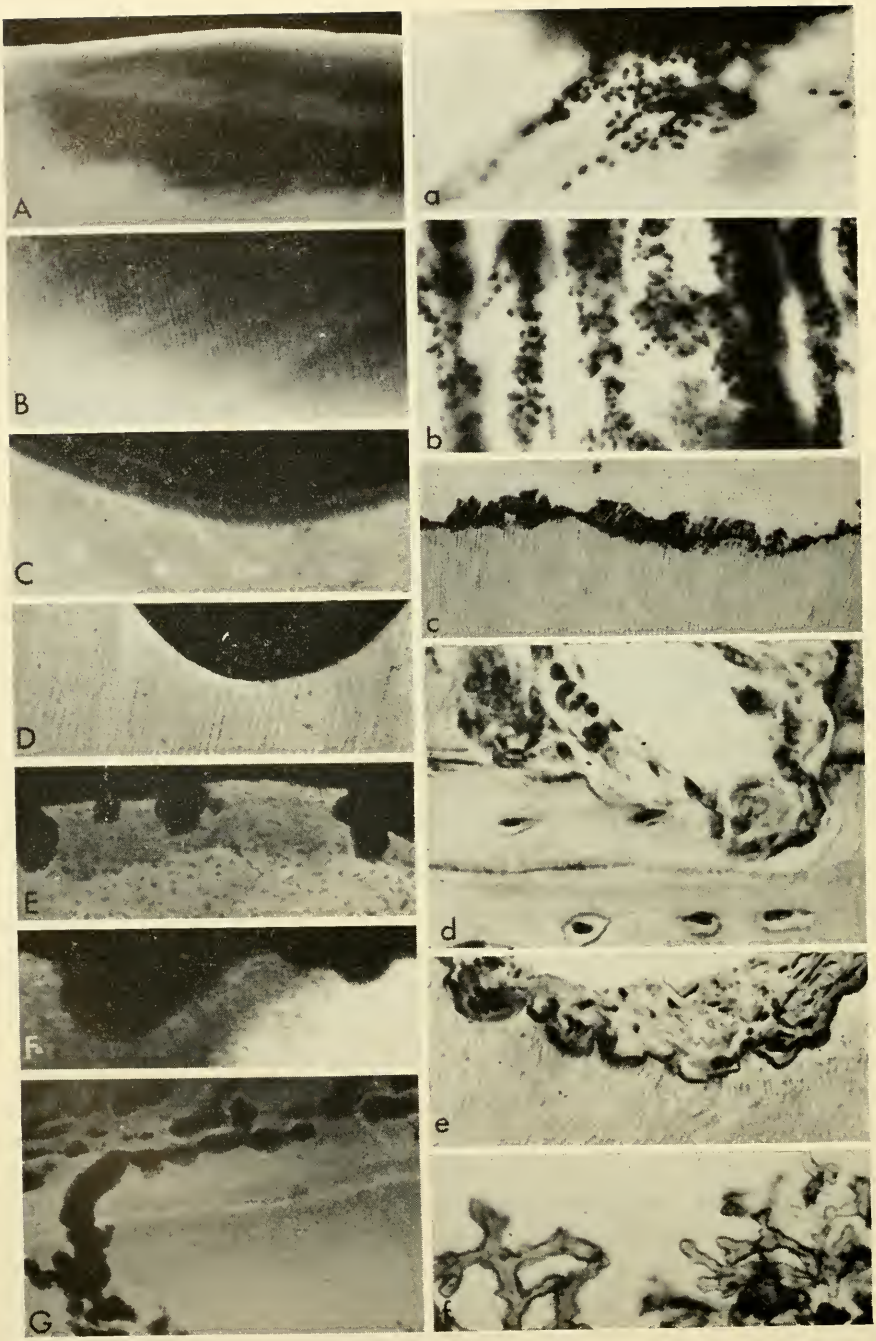


FIGURE 30

to both inorganic and organic elements involved in the fluid-solid equilibrium of the mouth (Sognnaes, 1957*b*).

From a mechanical point of view it is known that self-cleansing surfaces of enamel can tolerate a certain degree of grinding and polishing. In one clinical report Rushton (1957) has indicated successful practical results with rather extensive mechanical "remodeling" of the enamel for cosmetic purposes.

Thus, there are at least preliminary observations to suggest that the original intact enamel surface may no longer be considered inviolate. If it is partially destroyed—within a certain degree and depth—there appears to exist a limited capacity for reconstitution to clinically normal quality and appearance. This chemical repair appears to operate whether the original enamel surface has been destroyed by chemical action (as in our own experiments), by pathological demineralization (as in incipient caries), or by mechanical abrasion (as in cosmetic "remodeling" of malshaped or malposed teeth).

Concluding Comparisons and Summary

Having made references to various types of hard tissue destruction over and beyond dental erosion—the principal theme of this presentation—it seems appropriate in a few concluding paragraphs to present a brief summarizing comparison between the various types of dento-alveolar destruction processes from the point of view of microradiographic and histopathological characteristics. This comparison is presented in parallel fashion in Fig. 30A to G (microradiographs of ground sections) and Fig. 30a to f (photomicrographs of decalcified sections), and diagrammatically summarized in Fig. 31.

Fig. 30. Microradiographs of ground sections (left row, A to G) and photomicrographs of decalcified sections (right row, a to f) from dento-alveolar structures subjected to various mechanisms of hard tissue destruction: A, a, enamel caries; B, b, dentin caries; C, c, dentinal erosion; D, dentinal abrasion; E, d, alveolar bone resorption; F, e, dentin and enamel (e) resorption; G, f, cementum and dentin boring canals produced by postmortem saprophytes. For comparative comments and conclusions see text.

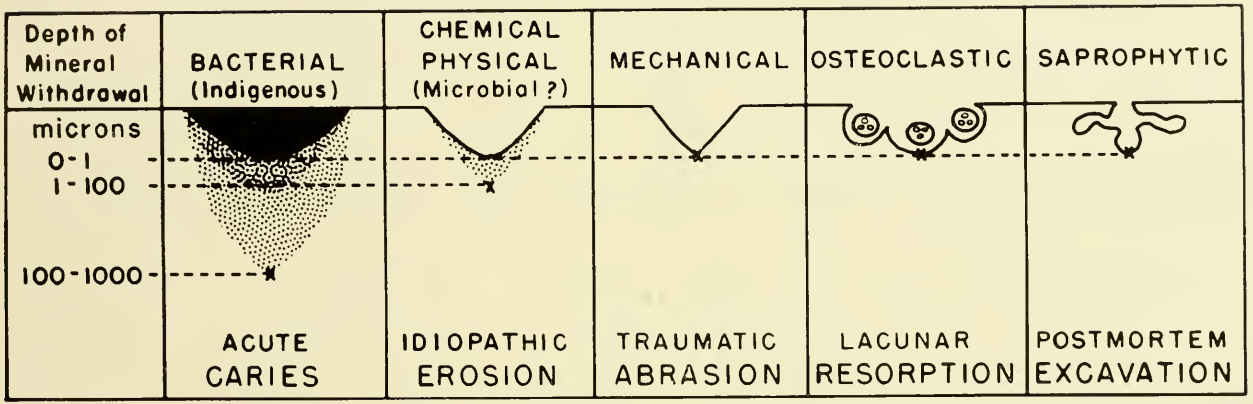


FIG. 31. Diagram summarizing the results of the microradiographic analysis of various types of hard tissue destruction to indicate the environmental agents and the relative extent of subsurface demineralization gradients, which decrease significantly from caries and erosion to resorption and postmortem destruction of the calcified tissues. (From Sognnaes, 1959.)

Dental Caries

The pattern of the initial carious lesion is intimately related to certain structural pathways within the dental hard tissues that are exposed to the external environment of the mouth. The destructive process is pathologically specific. One of the most distinctive microstructural features of dental caries is a deep subsurface demineralization of about 1000 microns, whether it be enamel (Fig. 30A) or dentin (Fig. 30B), prior to invasion of the tissues by oral microorganisms (Fig. 30a) and proteolytic disintegration of the organic matrix (Fig. 30b). In this respect, dental caries is markedly different from other types of hard tissue destruction (see Fig. 31), in which subsurface demineralization and preformed structural pathways do not appear to be by far so significantly involved in the pathogenesis of the lesions. The relatively higher resistance to demineralization observed within a narrow surface zone of incipient enamel caries (Fig. 30A) may be explained in part by the higher fluoride content now known to be present in this zone and in part by what has been suggested to be a protective interaction of organic surface films deposited on the inorganic phase of the tooth substance from the adjacent liquid phase of saliva.

Dental Erosion

The gross distribution and morphological variety of idiopathic dental erosion defects preclude a simple mechanism of mechanical wear as a primary cause of this disease.

Microradiographic observations of ground sections have shown at certain stages a minute subsurface demineralization gradient (Fig. 30C) never seen to exceed 100 microns in depth (i.e., less than one-tenth of what can be found in incipient caries). Consequently, one cannot, in beginning erosion, detect any change in opacity by naked-eye inspection so as to distinguish the lesion from simple abrasion, in which there is no subsurface demineralization at all (Fig. 30D).

Histological examination of stained decalcified sections suggests that dental erosion is not necessarily devoid of surface deposits of bacteria-laden mucus (Fig. 30C), though the lesion may look clean to the naked eye. Occasionally, these superficial deposits may be

separated from the tooth surface by a thin cuticular membrane, which stains orthochromatically with toluidine blue, presumably originates from saliva, and possibly may be of protective significance.

Tentatively, one may suggest that a three-stepped sequence of events may be involved in the pathogenesis of "idiopathic" dental erosion: (1) primary absence or secondary loss of some protective salivary organic coating on the tooth surface; (2) drainage of minerals from the unprotected and, hence, more soluble peripheral tooth substance by some decalcifying agent present in or brought into contact with the oral environment; and (3) destruction of this superficially decalcified tooth surface through biochemical and biophysical influences or even simple mechanical friction by lip, cheek, tongue, food, or toothbrush.

Resorption

On the basis of what has been learned from dento-alveolar resorption processes (Fig. 30E and F), one cannot point to the content and distribution of cellular elements (Fig. 30d and e) or to any consistent proportion of inorganic and organic ingredients to account for the origin of the multinucleated osteoclasts, on the one hand, and the susceptibility to resorption on the other. It is suspected, therefore, that changes in the environment adjacent to the disintegrating surfaces must be explored for factors that induce the virtually simultaneous removal of organic and inorganic elements of these tissues in the process of resorption (Fig. 31).

Postmortem Destruction

Postmortem changes have been observed in human teeth of prehistoric, ancient, and recent times originating from Palestine, Egypt, Greece, Iceland, Norway, and Central America. The histological observations indicate that the commonest postmortem change consists in large irregular boring canals which penetrate the dentin and cementum, leaving sharply defined margins with no evidence of any gradients in demineralization (Fig. 30G). The microscopic pattern of the postmortem destruction is suggestive of an invasion by fungi (Fig. 30f). Postmortem changes were rarely found in the enamel and were limited to localized areas of erosion of the enamel surface.

Remineralization

The theoretical concept of redeposition of minerals in partially demineralized enamel has been reappraised on the basis of recent observations on radioisotope exchange reactions between enamel and saliva in primate teeth. Experimental application of acid to the enamel surface of teeth *in vivo* resulted in demineralization in depth beyond what can be detected by gross inspection or by surface replica techniques. In the living animal the demineralization has a definite preferential pattern along the incremental lines of Retzius before grossly visible loss of surface contour. In the normal animal these areas of demineralization appear to be prone to redeposition of minerals, demonstrable within a week or two. However, despite the regained high microdensity, these remineralized areas appear to contain an organic substance which has a greater dye-binding capacity than intact mature enamel. It is believed that enamel in which minerals are redeposited following experimental demineralization is not completely reconstituted to its original form, and that it would be inappropriate to label this mechanism as a "genuine healing process."

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5

Attrition of the Hypsodont (Sheep's) Tooth

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EXCESSIVE wear of incisor teeth of sheep is an economic problem in New Zealand which has become aggravated during the past thirty to forty years. Teeth wear much more rapidly on improved pasture, chiefly rye grass (*Lolium perenne*) and white clover (*Trifolium repens*), than on the fine native grasses of low carrying capacity. Excessive wear of sheep's teeth is now becoming a problem in other countries where extensive methods of farming are being replaced by intensive practices through the introduction of improved pastures. Most of the results reported in this paper were obtained with Romney sheep, the predominant breed in New Zealand.

Since the problem is of regional occurrence—certain districts being noted for sheep "not holding their mouths"—first impressions were that it was of nutritional origin, related to soils and pasture composition and possibly to defective mineral metabolism. It was eventually concluded that the cause is not nutritional in the generally accepted sense, however, since the defect is quite different from caries; and there were no skeletal abnormalities, which are usually associated with deranged calcification. The wear is also distinct from abrasion caused by the presence of foreign gritty particles in the food. The various factors concerned compare and contrast interestingly with those responsible for dental caries in man (Barnicoat, 1957).

General

The eight incisors ("teeth") of sheep (actually six incisors, the two corner teeth being canines) are confined to the mandible and bite against a pad in the upper jaw. The molars ("grinders"), twelve in each jaw, are mainly used in rumination and are not of particular interest to this discussion.

Deciduous teeth are shed at 12 to 15 months, after which four pairs of permanent incisors erupt in due order when the sheep is about 15, 24, 33, and 48 months of age. It is then "fresh full-mouthed." The first pair (centrals) often contain as much tooth substance as the rest, and their rate of wear controls that of the others.

The hypsodont tooth consists of a crown covered with enamel, and when it is first cut only about half of the crown appears above the gum (Fig. 1). The remainder of the crown erupts as the animal grows and matures, mostly during the summer and autumn.

If the tooth is not subject to wear, it retains its "chisel" edge, and may attain a length of 20 mm or even more when the animal is 5 years old.* Slight wear gives teeth with flat biting surfaces, which are preferred by most farmers. When wear is excessive, however, the unerupted crown responds by emerging from the gum more rapidly than normal. When all the crown has appeared the tooth may wear eventually to a "gummy" state. On the other hand, teeth of older sheep not fully erupted may actually lengthen when they are transferred from sparse to generous conditions of feeding.

Histological studies show that in the earliest stages of wear, when the thin layer of enamel on the incisal surface has just worn down, dead tracts in the dentine appear between it and the apex of the pulp chamber, and the deposition of secondary dentine is initiated. Sheep's teeth have a surprising facility for forming secondary dentine, which plugs the pulp canal as it is paid out of the gum with the tooth as it wears (Figs. 2 and 3). Incisal surfaces of older sheep may consist entirely of secondary dentine. The secondary dentine is relatively soft, according to the Tukon Hardness Tester, as shown

* Hill country sheep are graded and sorted when 5 years old, the condition of the mouth being an important factor.

in the accompanying table. Yet when mouths are nearing the "gummy" stage the plugs of secondary dentine appear to be the most resistant part of the tooth toward wear.

	Knoop hardness numbers (air-dried teeth)	
	Average	Range
Enamel	256	(220–288)
Dentine	49	(41– 56)
Secondary dentine	25	(13– 32)

During the course of this investigation, which involved the examination of thousands of sheep's mouths, dental caries was never detected, though the mouths usually contained much food debris and fibrous portions lodged between the teeth (Fig. 3).

The sheep's incisor tapers from the incisal surface and therefore narrows as it wears, which causes gaps to appear between teeth (Figs. 1 and 3). This gives an appearance of spreading, which was not, however, confirmed by measurements of casts of mouths of individual "full-mouthed" ewes taken at different ages.

Factors Responsible for Wear in Sheep's Teeth

These may be classified as (1) managemental (farming) practices, (2) anatomical (hereditary) differences in individual mouths, and (3) nutritional factors.

Managemental Practices

Changes in farming practices may be of paramount importance in overcoming the problem for individual farmers. Suffice it to say that the type of pasture, as well as its length as controlled by grazing stock, has great influence on rate of wear of sheep's teeth.

Anatomical (Hereditary) Defects

Short and long lower jaws* are common faults in sheep. Only after studying photographs of the mouths of over 100 individual

* The terms *undershot* and *overshot* are avoided since they have opposite meanings in different countries.

FIG. 1. Central incisors from 18-month-old (two-tooth) ewes (× 3). The gum level is shown by grass stain (arrows) in A and C. The junction of enamel-covered crown and root is marked with pencil, and the unerupted part of the crown lies between arrow and pencil mark. A contained approximately twice as much "useful tooth substance" as C; B, a wide tooth, though rather less in weight than A, is a better type of tooth.

sheep taken at intervals until they attained the age of 5 to 6 years did other well defined faults become evident. They are: narrow teeth, "splayed" or "fanned" central incisors, overlapping central incisors, deep "V" in central incisors, and cleft palate.

All these abnormalities are also detrimental to the lasting quality of the teeth, and it is unusual to find only one type of fault present in a defective mouth. Moreover, certain defects in 5-year-old mouths are totally different in appearance at the early stages; for example,

FIG. 2. Longitudinal transparent section of sheep's tooth, about half worn, showing plug of secondary dentine. Note thicker layer of enamel on labial (top) surface. (× 5.)

overlapping central incisors usually develop into a deep "V." Except in grossly defective mouths, the permanent teeth are not necessarily similar to the deciduous; and the mouths of twins are usually unrelated in type. Short lower jaw is the commonest mouth defect in New Zealand sheep. This type of mouth wears down quickly, and even the presence of this defect to only a mild extent, not usually recognized as a fault by farmers, increases the rate of wear.

These defects are probably all of hereditary origin and some are congenital. The laws governing the inheritance of shape and alignment of sheep's teeth are, as yet, little understood. The faults are widespread, but by adopting a long-term policy of selecting desir-

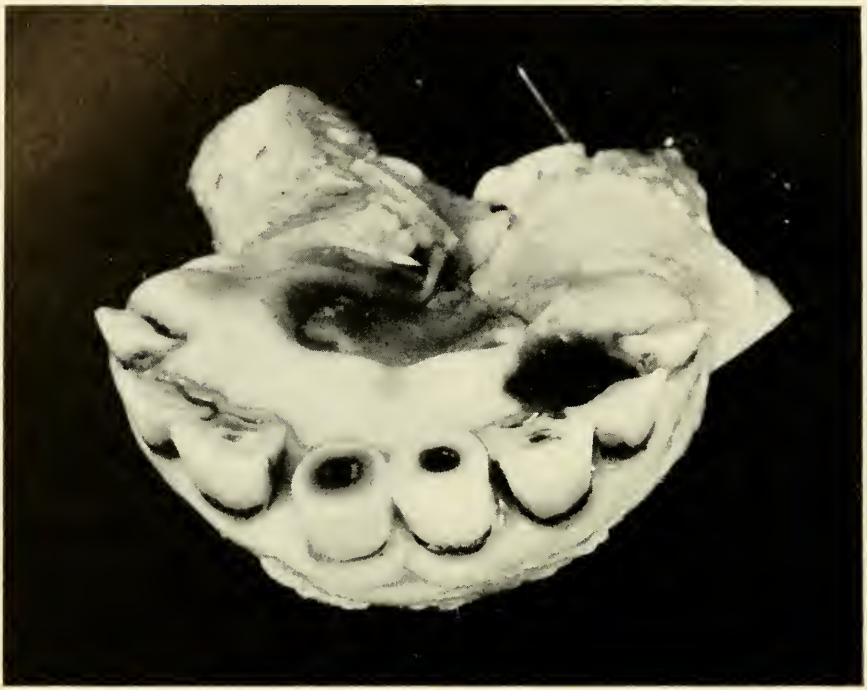

FIG. 3. Excessively worn mouth of 4-year-old ewe, showing caries-like appearance due to staining of plugs of secondary dentine.

able mouths of breeding ewes and, more important, of rams, it is possible to "grade up" gradually the quality of the mouths of the whole flock.

An interesting feature is that in flocks of sheep noted for sound teeth, these faults may merely be latent in the animals while grazing "good teeth" country. When the sheep are transferred at an early age to "improved" high-yielding pastures, a surprising range of defective mouths usually develops as they mature.

Nutritional Factors

Results of nutritional trials with sheep emphasize the importance of adequate intakes of calcium and phosphorus.

Franklin (1950) found that diets (grains) which were deficient, particularly in calcium, caused a general stunting of the whole skele-

tal structure, as well as irregularities and gaps in the molars. McRoberts and Hill (1962) concluded that "diet deficient in calcium had a greater effect on the teeth than on the skeleton, while the diet deficient in phosphorus and in Vitamin D had a greater effect on the skeleton than on the teeth."

Recommended minimum adequate requirements of calcium and phosphorus for sheep (Natl. Research Council Natl. Acad. Sci. (U. S.), 1949) as percentage of dry matter are:

	Ca	P	Ca/P
Growing lambs	0.18	0.15	1.2
Ewes	0.20–0.23	0.18–0.24	1.2–1.6

The average composition of herbage eaten by New Zealand sheep in relation to extent of wear of teeth is recorded in Table I. The intake of calcium and phosphorus is satisfactory by accepted standards; and, in fact, the better teeth are found in animals ingesting herbage with the lower mineral content.

No relationships between quality of sheep's teeth and deficiencies or imbalances of microelements (i.e. the biologically important copper, molybdenum, cobalt, iron, fluorine, iodine*) were established; nor is there any apparent relationship with geological formation or soil type. There is no evidence to support the theory that vitamin A (as the provitamin carotene) or D is implicated, and the water-soluble vitamins are believed to be more than amply provided by the rumen microflora (Barnicoat, 1959).

Analyses of enamels and dentines of teeth of New Zealand sheep are recorded in Table II. Their stoichiometric relationships are given in Table III. Figures vary little, and are not influenced by the quality of the mouth. These analytical results are similar to those reported by numerous workers for human teeth (Leicester, 1949).

Spectrographic analyses of enamel and dentine for 19 microelements of biological importance, including aluminium, cobalt, copper, iron, molybdenum, silicon, vanadium, and zinc, showed no differ-

* Paradontal disease, chiefly affecting molars, is associated with selenium deficiency (Hartley and Grant, 1961).

TABLE I. Extent of Wear of Teeth of 5-Year-Old Ewes[a] in Relation to Average Composition of Herbage Eaten

Herbage species	Typical 5-year mouth			Average chemical composition (per cent dry matter)					
	Description	Av. length central incisors (0.1 in.)	Av. rate of stocking (ewes/acre)	Crude protein	Crude fiber	Ratio polysaccharide[b] to crude protein	Ca	P	Ca/P
High-production perennial rye grass and clovers from top-dressed pastures	Poor or "gummy"	2.5	5–7	30	16	1.8	0.80	0.43	1.9
Medium-production grasses from top-dressed fat-lamb "easy" hill country	Poor to fair	3.5	2–4	21	22	3.0	0.45	0.33	1.4
Native grasses from hill country and other parts used for sheep breeding	Good, excellent, slightly worn	4.5	1–2	9	30	8.6	0.29	0.23	1.3
Weeds and scrub tips from "hardest" sheep-breeding country	Practically unworn	6	<1	27	18	2.4	0.32	0.43	0.9

[a] Referred to as 5 years old in this article, as in practice, but actually 5½ years. The permanent teeth are cut when sheep are about 1½ years old.

[b] Polysaccharide = nitrogen-free extract and crude fiber.

TABLE II. Chemical Composition of Sheep's Teeth

(Actual range of values shown in parentheses, number of samples in square brackets. Results in parts per cent dry basis, except fluorine, parts per million)

Constituent	Enamel			Dentine		
	Averages	Teeth with least wear	Teeth with most wear	Averages	Teeth with least wear	Teeth with most wear
Calcium[a]	37.6 (37.1–38.2) [20]	37.6 [6]	37.5 [6]	35.05 (34.3–35.6) [10]	35.0 [4]	35.2 [4]
Phosphorus[a]	18.8 (18.2–19.2) [20]	18.6 [6]	19.0 [6]	18.0 (17.9–18.4) [10]	18.25 [4]	17.9 [4]
Ratio calcium/phosphorus[a]	2.0 (1.93–2.06) [20]	2.02 [4]	1.97 [4]	1.95 (1.90–1.98) [10]	1.92 [4]	1.96 [4]
Magnesium[a]	0.28 (0.24–0.30) [20]	0.28 [8]	0.28 [8]	1.08 (1.02–1.16) [5]	—	—
Sodium[a]	0.55 (0.51–0.68) [20]	0.57 [8]	0.54 [8]	0.71 (0.68–0.74) [5]	—	—
Carbonate[b] (as CO_2)	2.53 (2.30–2.89) [25]	2.52 [6]	2.54 [7]	2.79 (2.45–3.14) [25]	2.80 [6]	2.79 [7]
Citrate[b] (as citric acid)	0.057 (0.044–0.077) [25]	0.061 [5]	0.057 [5]	0.76 (0.68–0.83) [20]	0.74 [5]	0.76 [5]
Nitrogen[c]	0.04 [22]	0.04 [5]	0.06 [5]	3.34 (2.85–3.62) [22]	3.29 [5]	3.51 [5]
Fluorine[a,d]				149 (43–290) [20]	157 (43–280) [8]	155 (62–290) [7]

[a] Ash.
[b] Fat-free dry matter, separated by flotation method (sp. gr. 2.70) (Manly and Hodge, 1939).
[c] Similar treatment (sp. gr. 2.85).
[d] Ground mixed whole teeth, mainly dentine (ppm).

TABLE III. Stoichiometric Relationships in the Enamels and Dentines of Sheep's Teeth

(Dry, protein-free basis)

1. Representative sample from 20 full-mouthed sheep.
2. Central incisors, recently erupted, from 18-month-old ewe.
3. Teeth from 5-year-old sheep on a farm noted for outstandingly good mouths.
4. Teeth (5-year-old ewes) with highest CO_2 content.
5. Teeth (5-year-old ewes) with lowest CO_2 content.

	Enamel					Dentine				
Member	1	2	3	4	5	1	2	3	4	5
Calcium (%)	37.6	37.7	37.2	37.6	37.6	35.05	34.3	35.6	35.05	35.05
Phosphorus (%)	18.8	19.0	18.15	18.8	18.8	18.0	17.65	18.15	18.0	18.0
Carbon dioxide (%)	2.57	2.42	2.40	2.94	2.34	3.67	3.65	3.65	3.83	3.31
Calcium (mM %) × 100	93.8	94.0	92.8	93.8	93.8	87.4	85.6	88.8	87.4	87.4
Phosphorus (mM %) × 100	60.6	61.2	58.5	60.6	60.6	58.0	56.9	58.5	58.0	58.0
Carbonate (CO_3) (mM %) × 100	5.84	5.50	5.45	6.68	5.32	8.34	8.29	8.29	8.70	7.52
Ca : PO_4 ratio[a]	1.55	1.54	1.59	1.55	1.55	1.51	1.50	1.52	1.51	1.51
PO_4 : $2CO_3$ ratio[b]	5.2	5.6	5.4	4.5	5.7	3.5	3.4	3.5	3.3	3.9

[b] The PO_4 : $2CO_3$ ratio = n in the formula for apatite $(Ca_3[PO_4]_2)_n(CaCO_3)_{1.0}$ when the molecular ratio [a] Ca : PO_4 is 1.50. This is equivalent to a weight ratio Ca/P of 1.94.

ences except for silicon. The silicon content of dentine of the most worn teeth was twice that of dentine from relatively unworn mouths.

Fluorine is the only element showing wide variations. In 50 samples of mixed (whole) teeth from 21 farms, fluorine ranged from 28 to 320 parts per million, averaging 149 ppm, a value similar in order to that for human teeth. It might be expected that teeth of low fluorine content would be the most rapidly worn, since human teeth of this type are susceptible to caries; or that teeth of high fluorine content might appear bleached or mottled, with irregularities in shape, as is typical of fluorosed sheep's teeth in Australia (Peirce, 1938), although fluorine contents never approached those of even the most mildly affected Australian samples. Such defects were not observed, however, nor were there any indications that the proportion of fluorine in New Zealand sheep's teeth influences their tendency to wear. If there is any such tendency, it is obscured by other factors.

Since other constituents vary so little, the wide differences in fluorine may seem surprising especially since the fluorine content of New Zealand soils shows little variation (Gemmell, 1945). It would appear that fluorine in sheep's teeth is derived from that applied in phosphatic manures, which are reported to contain about 1 per cent fluoride. For example, teeth from an experimental area heavily top-dressed for 14 years contained on the average 290 ppm fluorine, whereas teeth from the neighboring farm on the same type of soil, little top-dressed, contained only 123 ppm. The lowest values for fluorine appear to be associated with farms which have not been artificially manured. These values are low in comparison with those reported overseas, both for sheep's and for human teeth, but some of the best teeth came from these properties.

Because of lack of chemical evidence of differences between diets, or in the composition and histological appearance of teeth of widely different types, it is concluded that the problem is not attrition of nutritional origin in the usually accepted sense.

Before discussing this investigation further, the peculiar aspects of the New Zealand problem of excessive wear in sheep's teeth should be restated: The wear occurs in teeth of sheep grazing luxurious, highly productive, "improved" pastures. The animals, judging

by their appearance and productivity, are healthy and thriving. Excessive wear is not found when sheep are carried on low-producing, wiry native grasses (Table I). Wear does not appear to be caused by simple abrasion; e.g., no undue wear is observed in mouths of sheep maintained under hard conditions on sandy or pumice country, although these abrasive siliceous materials are splashed by rain and carried by wind onto the pasture plants.

The appearance of worn teeth and the type of wear indicate that the wear is caused by both erosive and physical effects. The erosive action was first believed to be brought about by compounds in the herbage sap complexing the calcium of the apatite (essentially tricalcium phosphate) which constitutes the inorganic, and major, fraction of the tooth substance. In high-yielding pasture, chelating agents such as acids of the Krebs cycle, amino acids, and phosphorylated compounds of carbohydrate metabolism would occur in increased concentrations, especially in the meristematic tissues of the actively metabolizing young leaves.

Removal of the superficial layers of the incisal surface of the tooth could be further facilitated by the mechanical action of the harder constituents of grass, such as the fiber, which at each bite is pulled and stretched between the incisal surface and the pad of the upper jaw before being nipped. "Improved" pastures are usually rich in perennial rye grass (*Lolium perenne*), which is generally regarded as particularly destructive to sheep's teeth. Mechanical tests also show that rye grass is considerably tougher than others, especially native grasses. Baker *et al.* (1959), in Australia, concluded that the opal phytoliths in plants could play an important part in the abrasion of sheep's teeth.

In order to study the effect of organic acids, polished sections of air-dried sheep's teeth were exposed to 0.05 M solutions of organic acids, pH 6.1, for 20 hours at 10°C, these concentrations of acid and pH being similar to those of herbage press juice. Hardness tests were made with the Tukon Hardness Tester before and after treatment. Results are recorded in Table IV. These results indicate that in solutions similar in properties to grass press juice the four acids of the Krebs cycle readily attack teeth. Malic acid, the predominant organic acid in grass sap, is even more active than citric, used in

TABLE IV. Effect of Acids Occurring in Plants on the Hardness of Sheep's Teeth

(Initial average Knoop Hardness Number: dentine, 47; enamel, 203)

Acid	Chemical structure	Per cent reduction in hardness after treatment	
		Dentine	Enamel
Organic acids of Krebs cycle			
Glutaric	C_5 dibasic	27	100[a]
Citric	C_6 tribasic-monohydroxy	62	58
Succinic	C_4 dibasic	80	51
Malic	C_4 dibasic-monohydroxy	35	100[a]
Amino acid			
Aspartic	C_4 dibasic-monoamino	10	4
Water (control)	—	−6[b]	4

[a] Too soft to measure.
[b] That is, a slight increase.

most of these experiments. The presence of a hydroxy group did not consistently influence chelating activity of the acids, whereas the amino group (in aspartic) practically eliminated it.

Solutions of citrate equivalent in concentration to the organic acids in grass (chiefly malate and citrate), at pH's from 5.5 to 6.5, dissolve finely ground sheep's teeth, apatite, and tricalcium phosphate, especially at the lower pH values. Even acetate under similar conditions has an appreciable action, about one-fourth that of the citrate.

Grass and clover press juices, however, have little solvent action on these phosphatic substances. When the phosphatic substances, after treatment with grass press juice followed by washing, are further extracted with 50 per cent *w/v* ammonium sulfate, small amounts of calcium and phosphate, in excess of the controls, are dissolved. This indicates that the action of constituents of the grass juice has produced a weakening in the apatite lattice, thereby increasing the solubility of its constituent atoms.

Barnicoat and Hall (1960) pursued this investigation with the electron microscope, using a technique which in addition to facilitating the study of the microstructure of enamel and dentine enables

the intensity of etching to be recorded (Hall, 1959 and unpublished data).

Electron micrographs of the biting edge of worn incisors indicated that the dentine surface is subjected to both chemical and abrasive action. In order to study more precisely the theory that organic compounds in the herbage initially complex the calcium phosphate (apatite), experiments were made to compare the etching produced by fractions of juices of pasture plants on surfaces of fresh dentine polished smooth by metallographic techniques. The fractions examined were (1) organic acids from herbage, obtained by ion exchange, at their original concentrations and pH (6.0–6.5) for chelating action on the calcium; (2) cell fluids, obtained by ether cytolysis, containing in addition sugars, inorganic and organic phosphates, amino acids, and polypeptides, capable of chelating and dissolving calcium; (3) freshly expressed juices of grasses and clovers containing enzymes in addition to substances of fractions 1 and 2.

These studies have shown that fraction 3 produced the strongest etching, and indicated that this reaches a maximum during the lush spring growth. It was also evident that the central noncalcified core of the odontoblast process was etched by fresh grass juice at a faster rate than the dentinal matrix, and thereby released long lengths of the membranelike sheaths enclosing the process.

The results indicate that most of the etching is due to proteolysis and that such weakening of the bonding material of the tooth would facilitate its wear by attrition. Rouiller *et al.* (1952) and Rouiller (1956) have observed that an organic component bonding the particles of dentine is digested by plant proteinases such as papain. It is considered, therefore, that the digesting action of the proteinases of actively metabolizing leaves on the organic compounds of dentine could be an important factor contributing to the wear in teeth of grazing sheep.

Summary

Increased wear in incisors occurs in sheep grazing "improved," lush, highly productive pasture.

Mouths with hereditary anatomical defects wear most rapidly,

though such defects remain latent and even undetectable in sheep of similar breeding grazing "unimproved" pasture.

The hypsodont tooth lays down secondary dentine in the pulp chamber as the tooth wears. The incisal surface of worn sheep's teeth may eventually consist almost entirely of secondary dentine. Caries has never been observed in sheep's teeth during this investigation.

Results of extensive feeding trials, and considerations of chemical analyses of the soil and the pastures, have, so far, given no indication that the problem is nutritional in the generally accepted sense.

Constituents of actively metabolizing herbage which predispose sheep's teeth to excessive wear appear to be: (1) enzymes (proteinases) which attack the organic bonding material of the dentine; (2) acids which chelate calcium of the apatite. By these agencies tooth structure may become weakened and susceptible to attrition by the tougher constituents of the herbage such as fiber and opal phytoliths.

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6

Microstructural Changes in Early Dental Caries

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THE first attack of dental caries is usually upon the dental enamel, so that this paper is concerned with caries of the dental enamel. It will also be confined to human dental enamel.

If one commences with the clinical cavity in the tooth, which is the only clinically identifiable lesion of caries, one can recognize certain histological appearances in the enamel surrounding the cavity which are typical of enamel caries. These can be traced back through earlier stages and can be identified in lesions in which the enamel surface is still intact.

It has been known for many years that at least two distinct processes are involved in enamel caries. One of these is demineralization and the other is proteolysis or breakdown of the insoluble keratinous organic matrix of the enamel. So far as can be assessed by histological examination of decalcified sections of enamel caries, the proteolytic changes begin only at or about the time of the breakdown of the enamel surface. At this stage there is already considerable evidence of demineralization of the enamel, and if observations are confined to lesions showing intact enamel surfaces, the complications of proteolytic change and bacterial invasion are avoided.

In such lesions observed in Canada balsam or quinoline by trans-

mitted light, several zones can be identified (Fig. 1). Of these the principal ones are the body of the lesion, with the dark zone lying deep to it while the translucent zone lies deep to the dark zone though it is not always seen. Nishimura (1926) described, in addition to the three zones already mentioned, a first stage of attack

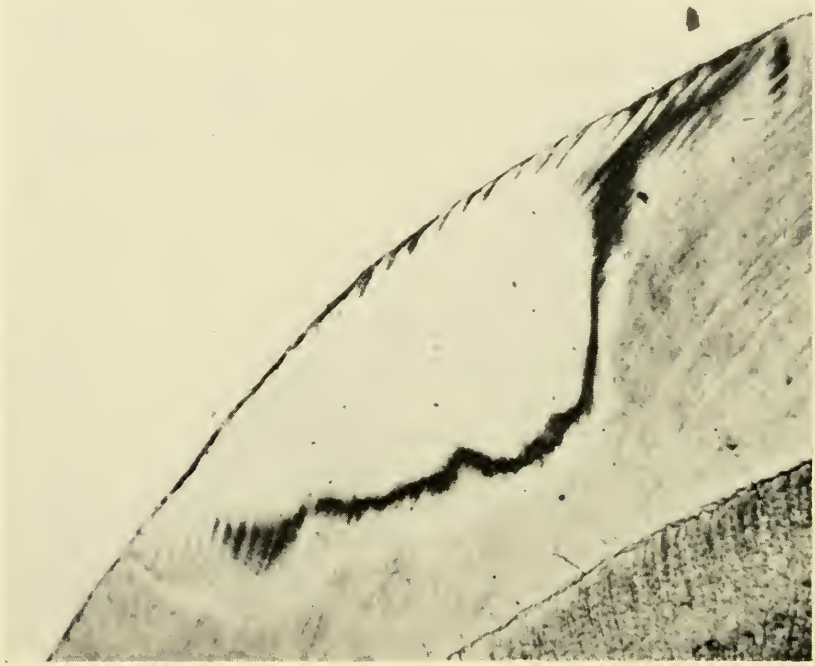

FIG. 1. Ground section of early caries of human dental enamel, mounted in quinoline. (× 52.) (Reproduced by kind permission of the editor of the *British Dental Journal*.)

at the deep margin of the translucent zone, and both he and Gustafson (1957) have placed some emphasis on a zone of translucence just superficial to the dark zone. Most observers agree that all these zones exist, but differ on the importance to be ascribed to them.

There can be no doubt that these zones represent successive stages in the attack of enamel caries, for the earliest lesions consist of a translucent zone only. After this stage the dark zone appears,

followed by the body of the lesion, and the intermediate stages occur in their appropriate places.

The most direct method of assessing demineralization is by microradiography of ground sections. This technique as used for dental purposes was pioneered by Applebaum (1932) and has since been used very successfully by Bergman and Engfeldt (1954), Guzman *et al.* (1957), Gustafson (1961), Darling (1956, 1958), and others.

By this technique it can be shown that the area of radiolucence or demineralization extends to the superficial border of the dark zone or possibly just into this zone (Fig. 2). It stops short of the enamel surface in most cases, leaving a zone of relatively unaffected enamel about 30 microns in width at the enamel surface. With care, greater detail can be shown within the lesion (Miller, 1958; Bergman and Engfeldt, 1954; Darling, 1958; Gustafson, 1961). All seem to agree that the earliest radiographic change which can be seen is related to the cross striations of the prisms, which are the incremental lines of growth of these structures. This is followed by the development of lines of radiolucence running parallel to the prisms, which are at first intersected by fine lines of radiopacity, again related to the cross striations (Fig. 3). These are later lost. In radiographs of sections transverse to the prisms (Fig. 4) the pattern of radiopaque material strongly suggests the outlines of the prisms with their "cores" demineralized, but no one has yet identified them as such. In sections longitudinal to the prisms the striae of Retzius or incremental striae of the enamel are grossly demineralized and form a dominant feature of many lesions. They appear to form the advancing edge of demineralization at the cervical margin of most interstitial lesions, giving a "sawtooth" effect at this edge (Fig. 2). Alternating with these striae are lines of relatively undemineralized enamel which some say (Darling, 1958) lie immediately beneath the striae of Retzius. In late lesions just before surface breakdown, radiolucent lines parallel to the striae of Retzius and continuous with those in the body of the lesion can be seen passing through the surface zone (Fig. 5), which until this stage is wholly radiopaque. Guzman *et al.* (1957) have described lines of radiolucence parallel to the prisms in the surface zone. They believe that the lines correspond to the prism cores, but they seem to occur in a relatively

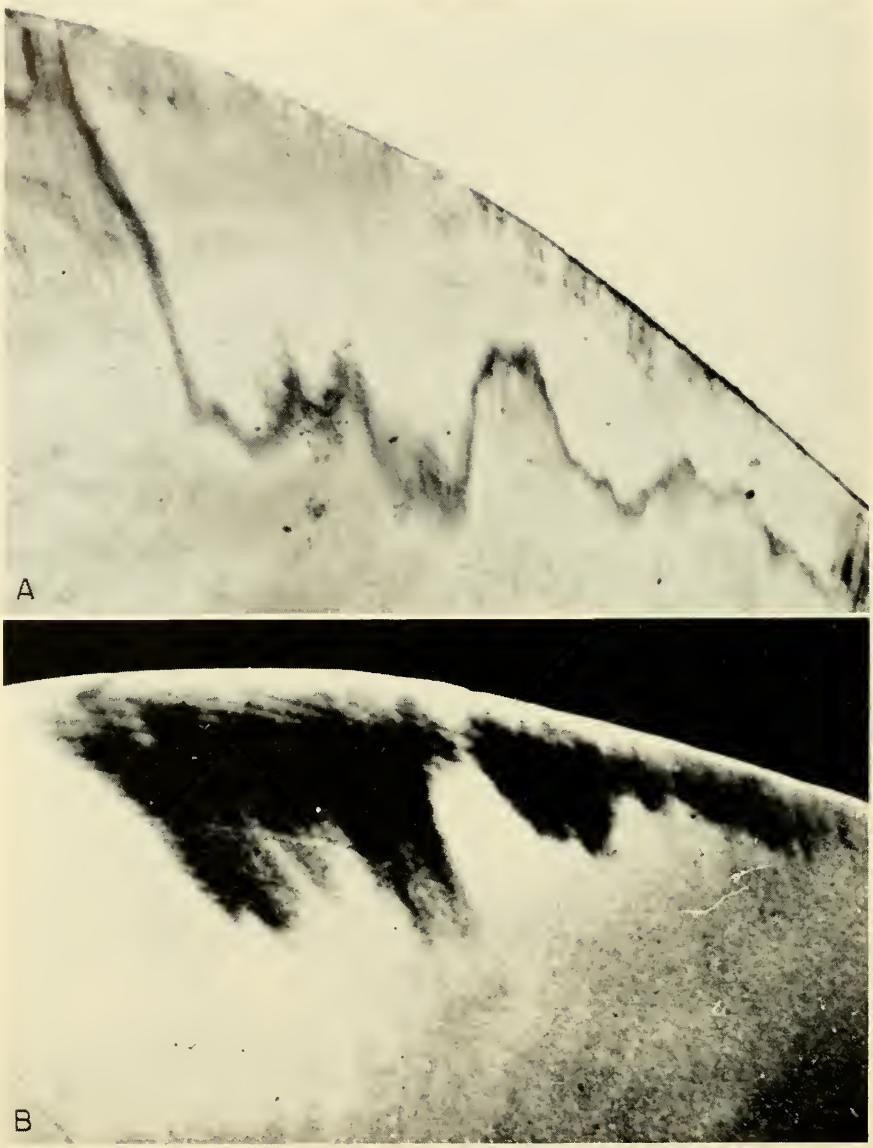

FIG. 2. Ground section of early caries of human dental enamel seen (A) in quinoline by transmitted light, and (B) by microradiography. (Both × 60.) (Reproduced by kind permission of the editor of the *Archives of Oral Biology*.)

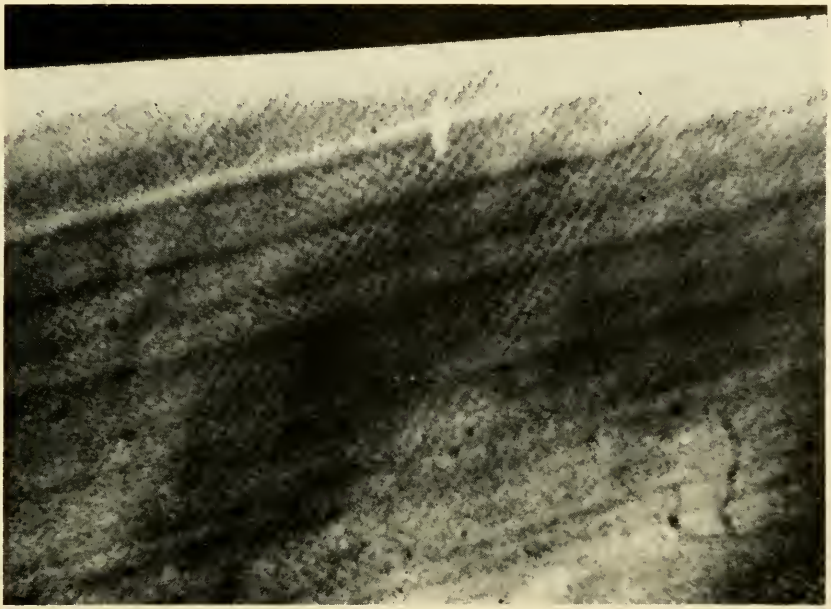

FIG. 3. Microradiograph of a ground section of early caries of human dental enamel cut parallel to prisms. (× 350.) (Reproduced by kind permission of the editor of the *British Dental Journal*.)

late stage just before surface breakdown. Mortimer (1962) has seen a similar appearance in the surface zone of occlusal lesions, where he finds that radiolucent striae of Retzius are rarely seen. Gustafson (1961) has pointed out that variations in the degree of mineralization of the enamel and its minute structures may play an important part in producing patterns of demineralization.

There can be little doubt that this pattern indicates a selective demineralization of certain structures of the enamel by caries, but whether this is caused by variations in the chemistry of these structures (Brudevold, 1961), variations in their physical properties, e.g. orientation of crystallites (Poole, 1962), variations in their accessibility to the demineralizing agent and its paths of entry (Poole, 1962), variations in the degree of mineralization (Gustafson, 1961), or variations in their inorganic-organic complex, is not yet clear. Each has its supporters.

One major point of difference between observers lies in the demon-

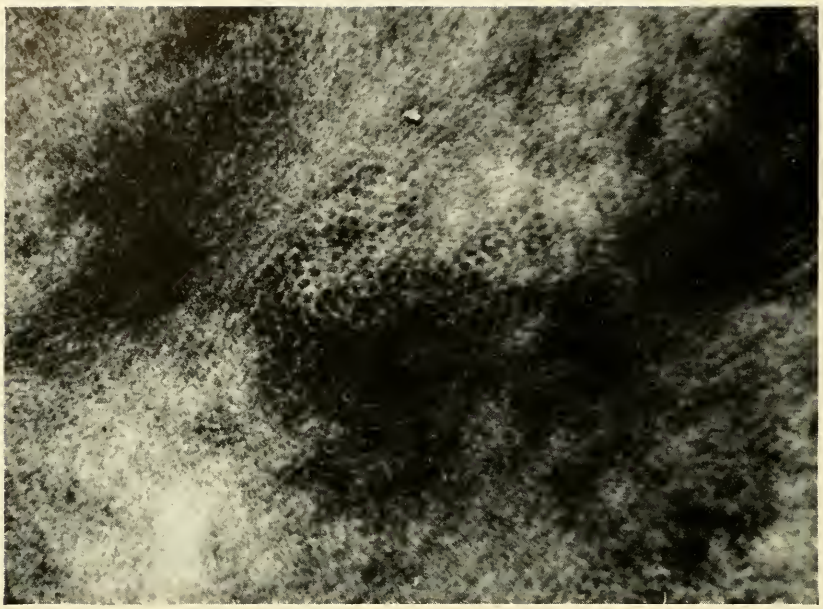

FIG. 4. Microradiograph of a ground section of early caries of human dental enamel cut transverse to the prisms. (× 350.) (Reproduced by kind permission of the editor of the *British Dental Journal*.)

stration by Gustafson (1961) of increased radiopacity in the translucent zone. From his evidence it appears that this can occur, though probably only in a relatively small proportion of lesions. He and Kostlan (1962) have also shown zones of highly mineralized enamel occurring occasionally within the demineralized area of the body of the lesion. The author has also observed these areas. Gustafson (1961) believes that there is reprecipitation of minerals in the translucent zone, and Kostlan (1962) has suggested that both these unusual features may arise from remineralization of the translucent zone during a resting period of caries, with by-passing of this zone when a new attack occurs. The explanation is attractive, but like so much in caries is difficult to prove.

For many years polarized light has been used for the examination of enamel and the carious lesion. It would be impossible to review all the literature on this subject in this paper, but the earlier work has been excellently reviewed by Gustafson (1945, 1957) and by

FIG. 5. Microradiograph of early caries of human dental enamel just before surface breakdown. (× 60.) (Reproduced by kind permission of the editor of the *British Dental Journal*.)

Schmidt and Keil (1958), who have done much to make this a valuable tool in dental histopathology.

In this field there now seems to be general agreement that, when examined in quinoline, whereas "normal" enamel is generally negatively birefringent with reference to the length of the prisms, the translucent zone is slightly more negative. From this point progressing toward the enamel surface, there is a fairly rapid transition through isotropic enamel to the dark zone, which is intensely positive, followed by a transition back through isotropy into the negative birefringence of the body of the lesion. The body of the lesion becomes less negatively birefringent toward the surface until just inside the surface the negative birefringence increases sharply to the surface (Fig. 6). At times a positive zone may be found just beneath the surface zone. In the late stages of the lesion the striae of Retzius in the surface zone often show positive birefringence. All these features have been excellently described by Gustafson (1957), Keil (1935, 1936), and others.

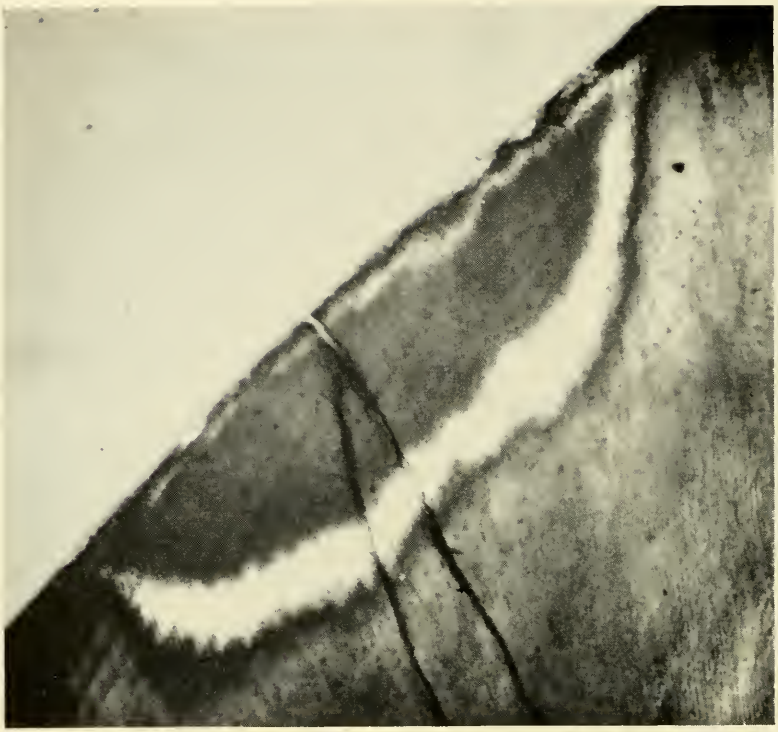

FIG. 6. Ground section of early caries of human dental enamel mounted in quinoline (R.I. 1.62) and seen by polarized light in the 45° position. (× 130.) (Reproduced by kind permission of the editor of the *British Dental Journal*.)

Their explanation has, however, caused considerable difference of opinion, which has concerned chiefly the translucent zone, the dark zone, and Nishimura's (1926) and Gustafson's (1957) third zone just superficial to the dark zone. Gustafson (1961) believed and has now shown by microradiography that the increased negative birefringence of the translucent zone can at times be due to increased mineralization. Usually, however, it is caused by a loss of the form birefringence present in normal enamel (Darling, 1958). Recent advances in the understanding of the birefringence of the carious lesion have arisen largely from the appreciation by Keil (1936) that spaces resulting from the carious process produce form birefringence to give the picture seen by polarized light in most mountants. This

formed the basis for the explanation of Darling's (1956) observation that the dark zone disappeared in aqueous media and his later demonstration (1958) that in an aqueous medium with a refractive index similar to that of enamel all the form birefringence of the carious lesion was abolished for all practical purposes. This permitted direct observations of the intrinsic birefringence of the enamel in the various zones as a measure of demineralization, and these were found to correspond to the general pattern of the microradiographic observations (Darling, 1956).

Using the same principles of form birefringence, it was found possible to estimate the amount of spaces in each zone (Darling, 1958). Later, with the demonstration by Poole *et al.* (1961) and Darling *et al.* (1961) that the spaces vary considerably in size so that they vary in their capacity to receive the molecules of mounting media, it became possible to assess the size of the spaces also, though this could only be expressed in terms of the molecules which they would admit. It is obvious that the new evidence disturbs many previous theories, but so far there has been little objection to it. The zones of dispute previously mentioned seem to be susceptible to explanation on the same basis, with the reservation that the translucent zone may at times be hypermineralized, when presumably it would show few if any spaces. On the basis of this evidence Darling (1961) has described normal enamel and the zones of the carious lesion (Fig. 7).

He states that "normal" enamel contains approximately 0.1 per cent of spaces of minute size penetrable only by molecules whose maximum size is similar to that of the molecule of water. These spaces are distributed chiefly in the interprismatic markings, cross striations, and striae of Retzius.

The translucent zone contains approximately 1 per cent of spaces, all of which are large enough to admit the molecules of octanol and quinoline. These spaces are also distributed chiefly in the interprismatic markings, cross striations, and striae of Retzius. They do not appear to grow by enlargement of those spaces found in normal enamel, but rather replace them abruptly.

The dark zone contains approximately 2 to 4 per cent of spaces of a great variety of sizes. The smallest are impermeable to all mole-

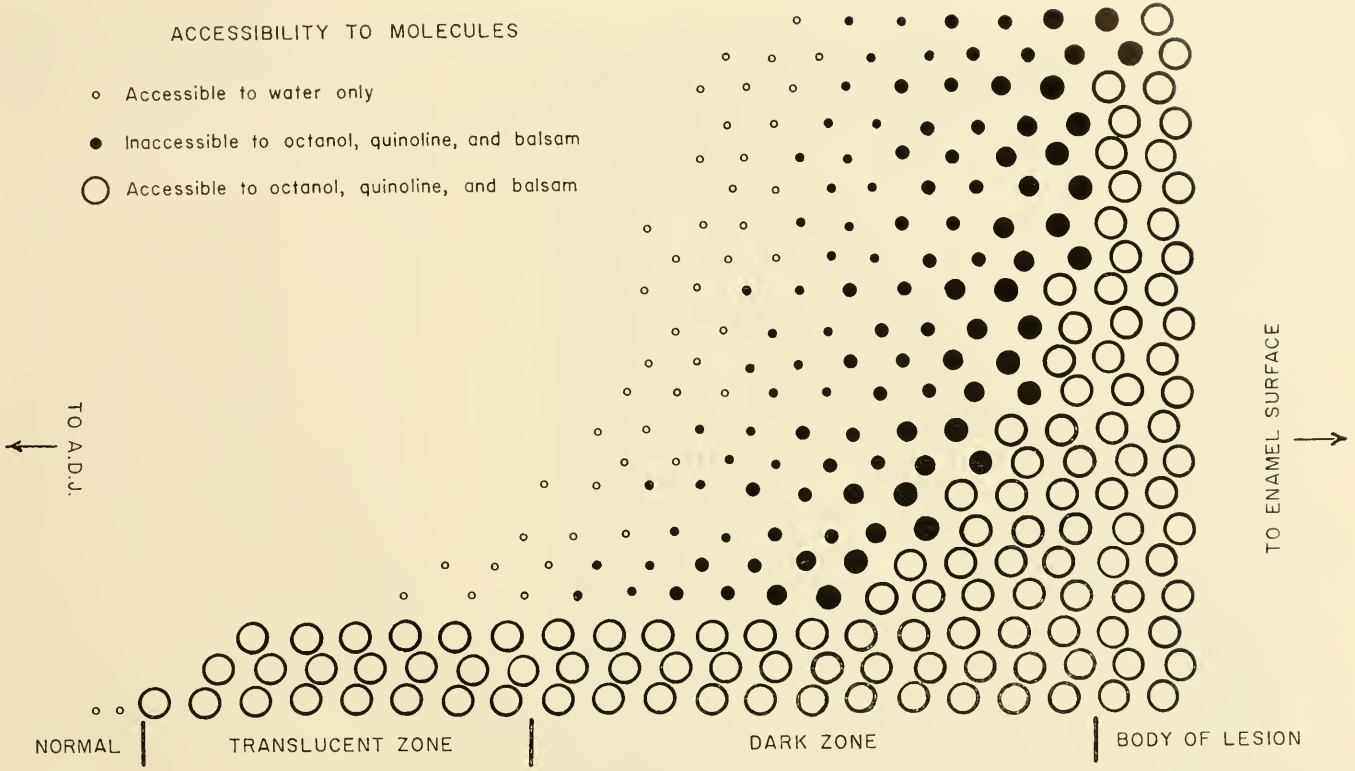

FIG. 7. Diagram showing the relative sizes and distribution of spaces found in the zones of the carious lesion in human enamel. *A.D.J.*, amelodentinal junction. (Reproduced by kind permission of the editor of the *Annals of the Royal College of Surgeons.*)

cules bigger than those of water, whereas the largest will accept octanol and quinoline with ease.

This zone is gradually transformed into the body of the lesion by the steady growth of the spaces in the dark zone. The body of the lesion contains at least 5 per cent of spaces and may contain up to 25 per cent or possibly 50 per cent or more. All these spaces are large enough to admit octanol and quinoline and possibly even larger molecules. Thus the zone of translucence described by Nishimura (1926) and Gustafson (1961) just between the dark zone and the body of the lesion would be explained in the same way and would be distinguished because the more highly demineralized parts of the body of the lesion probably have an altered refractive index which may produce some form birefringence in media of R.I. 1.62 (Crabb, 1962).

Using this knowledge of the spaces and their form birefringence, it is possible to demonstrate something of the pattern of spread (Fig. 8) (Darling, 1961). This begins as fine streamers spreading into the normal enamel along the so-called interprismatic markings. From these the cross striations are involved, and soon afterward the whole of the prism seems to be affected.

By combining the evidence from microradiography and observations of form birefringence, it is now possible to give a description of the various stages as follows.

The translucent zone shows no evidence of demineralization, though occasionally it may be hypermineralized. When the latter condition is not found, i.e. in the great majority of cases, this zone is characterized by the sudden development of large spaces in the sites where normal enamel has minute spaces (Fig. 7).

These spaces of the translucent zone persist in the dark zone but are overlain by the development of minute spaces penetrable only by the molecules of water. These grow throughout the dark zone until they become large spaces, big enough to admit octanol, quinoline, and balsam. When all the spaces reach this size the dark zone is transformed into the body of the lesion. The body of the lesion and to some extent the dark zone show demineralization. There can be little doubt that the minute spaces of the dark zone growing into the body of the lesion represent the results of demineralization, but

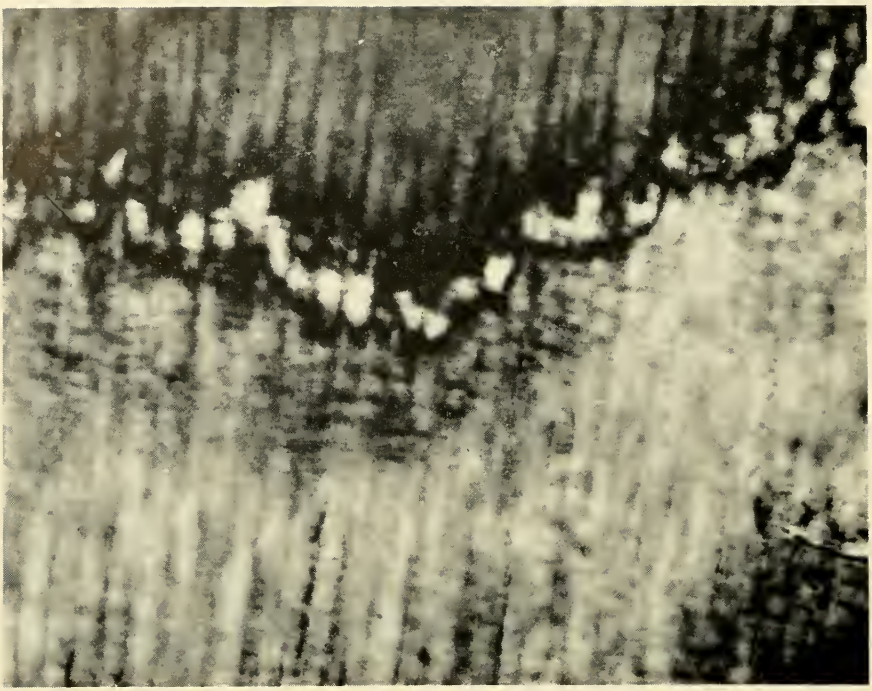

FIG 8. Ground section of early caries of human dental enamel mounted in quinoline and seen by polarized light in the 45° position. (× 750.) The illustration shows normal enamel at the lower border and passes through translucent zone and dark zone to the body of the lesion at the upper border.

it is not yet possible to say definitely what material is lost to produce the spaces in the translucent zone.

The explanations offered on the quality of the attack are to some extent modified by opinions on the precise structures involved and their nature.

Most workers now agree that there is a differential decalcification of certain structures. Most seem to accept that the pattern of attack, after the surface is penetrated, begins in the so-called interprismatic region, then involves the cross striations or something very like them, after which the prism becomes more completely involved, leaving rings of unaffected enamel which correspond in size to prisms. The precise location of these structures, however, is difficult to determine. All seem to agree that the surface zone, rings of enamel correspond-

ing in size to the prism cortices, zones beneath striae of Retzius, and lines parallel to the cross striations are not affected until late in the process of demineralization, but there is considerable difference of opinion as to why this should be.

Gustafson (1961) speaks chiefly of variations in the degree of mineralization as the cause of this differential demineralization. Brudevold (1961) has suggested that it is the chemistry of the structures and in particular the distribution of fluoride which is responsible. Poole (1962) believes that it may be largely a matter of the accessibility of the structures to the pathways of attack, and Little (1959) and Darling (1958) have suggested that it is related to differences in the organic matrix of the structures.

There seems no reason why all these possible causes should not play some part, but the marked difference in susceptibility between the surface zone and the deeper enamel makes it unlikely that this difference will depend to any great extent on the degree of mineralization or the accessibility of the structure to the pathways of attack. It seems that here at least the resistance to attack is more likely to depend on fundamental chemical or structural differences.

To recognize such differences as exist between these two groups of structures it is necessary to delineate them anatomically. Here again there is some difficulty. Most workers seem to agree that the early attack is at the margins of the structures known as prisms, but whether it is truly interprismatic or in the prism cortex or just outside or just within this structure, or even along spaces at the junction of the prism and the so-called interprismatic structure, seems to be open to argument. Similar arguments seem to apply to other stages in the anatomy of the attack.

Probably the best and most acceptable description which can be given at present is that the attack appears to take place first along the interprismatic or border region of the prisms, from which it spreads to involve the cross striations and later what seems to be the prism core, leaving stripes parallel to the cross striations, and, more obviously, what look like the cortices of the prisms, relatively unaffected.

In the surface attack there seem to be two possibilities which appear to conflict. Guzman *et al.* (1957) believe that entry is along

the prism cores, which can be seen on the outer surface of the enamel. Mortimer (1962) tends to agree so far as occlusal lesions are concerned, but it is very difficult to understand how such an attack can leave the surface zone apparently intact. It might be argued that what they see is the late spread of the attack back into the surface zone from its undersurface along the prism cores. The evidence in both cases is microradiographic. Darling (1956, 1958), on the other hand, both by birefringence and by microradiography, shows evidence of attack along the striae of Retzius passing through the surface zone into the lesion beneath it. Admittedly, the whole of the surface zone is involved in a process similar in some ways to that in the rest of the lesion, but it rarely passes the stages of translucent and dark zones before the whole of the surface breaks down. Even dark zones are rarely seen in the surface zone except along the striae of Retzius.

It seems tremendously important that the pathways of attack should be clearly identified on an anatomical basis.

As for the quality of the changes in the various zones, we know that the evidence obtained from the dark zone and the body of the lesion by various methods of examination suggests that the changes in these zones are produced by the progressive effects of selective or differential demineralization. The translucent zone, on the other hand, shows no evidence of mineral loss by microradiography or in birefringence. On the basis of work by Rowles (1955), Darling and Mortimer (1959), and Stack (1954), Darling (1961) has suggested that this zone may be caused by loss of organic material or carbonate. Darling and Mortimer (1959) have claimed that similar zones can be produced by removal of soluble organic material, though this work is being reexamined by them because of some doubts. The theory on which they base this work suggests that the selective decalcification of the enamel structures is related to the presence of predominantly "soluble" matrix in the "susceptible" structures and of "insoluble" matrix in the "resistant" structures. Both types of matrix are known to exist, but it is difficult to prove their precise distribution.

There can be no doubt that we are approaching a real understanding of the carious process in enamel, but the end is not yet and will

not be reached without much greater understanding of normal enamel structure.

Summary

The evidence on the structural changes in demineralization of enamel by dental caries is reviewed. The findings by microradiography and observations of intrinsic birefringence are in agreement and are accepted by most workers. By the use of form birefringence, spaces caused by the attack can be demonstrated. There is some difference of opinion on the paths of penetration through the enamel surface and on the precise detail and causes of the spread within the enamel, but it is generally agreed that there is a differential demineralization of the structures of the enamel. The zones of the enamel are identified and an explanation is offered for their appearances. Progressive demineralization is found in the dark zone and the body of the lesion and the intermediate zone, well in advance of histologically recognizable organic change. The translucent zone, though occasionally hypermineralized, usually shows a stage of attack without evidence of demineralization and because of this may represent a different type of attack. The nature of the attack in this zone is not clear, though suggestions are offered.

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7

Ultrastructural and Chemical Observations on Dental Caries

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CARIOUS lesions are manifestations of a complex disease phenomenon affecting the calcified tissues of teeth. It is known that numerous factors including the saliva will influence the course of the disease, and that the basic process by which lesions develop is generated by microbial metabolism in localized areas on external tooth surfaces. The acid environment thus created by metabolism of carbohydrates favors dissolution of dental tissues. Enamel and dentin do not respond to this injury by cellular regeneration or repair, but by a defensive obliteration of dentinal canals and development of secondary dentin between the lesion and the pulp. These formations at the same time constitute a barrier to the penetration of pulpal fluids into the lesion. On these grounds it can be assumed that the ultrastructural and chemical changes which have occurred in dental caries are mainly the results of the carious process with modifying influences primarily limited to factors associated with the fluid environment of the oral cavity.

The original ultrastructural studies on carious lesions of enamel and dentin to be summarized in the present report were carried out on zones of advanced destruction. This part of the tissue was chosen in order to observe maximal changes brought about by the carious

process. Similar zones were used for the chemical analyses of dentin, whereas technical difficulties introduced some variation in the sampling of enamel.

Observations on the ultrastructure and composition of carious lesions in various stages of development have been reported by other investigators. Höhling (1961) and Lenz (1961) noted morphological changes in crystallites from advanced carious lesions in enamel, and Scott and Albright (1954) compared the matrix of different zones within enamel lesions. In dentin, the distribution of mineral within zones of advanced lesions was studied by Takuma and Kurahashi (1962), and Bernick *et al.* (1954) noted the presence of cross-striated collagenous fibrils in carious matrix. These and other electron microscopic studies of carious tissues have recently been comprehensively considered by Helmcke (1962). Chemical studies by Coolidge and Jacobs (1957) of carious lesions of enamel with intact surfaces (white spot lesions) showed a decrease in the amount of calcium, phosphorus, and carbonate on a volume basis. Stack (1954) and Bhussry (1958) reported an increase in organic material in such lesions. In carious dentin, Manly and Deakins (1940) described three physically and chemically distinct zones and noted that volume loss of organic and inorganic constituents (from these zones) was associated with gain in moisture. The amino acid composition of carious dentin and its mucopolysaccharide content have been investigated by Armstrong (1961*a*, 1961*b*).

Comparable studies on sound tissues have recently been reviewed in detail by Brudevold (1962), Frank *et al.* (1960), and Johansen (1963, 1964). Even though areas of controversy and incomplete knowledge remain in this field of investigation, the information available provides a basis for comparison with conditions in altered tissues.

Materials and Methods

Fully formed permanent human teeth with active carious lesions and extracted in the city of Rochester, New York,* were used for both the chemical and the ultrastructural studies. For observations

* The water supply of Rochester contains 1 ppm of fluoride.

on carious enamel, lesions with and without break in surface continuity were selected. The ultrastructural studies were performed on areas of advanced destruction in both types of lesions. For the chemical analyses, lesions with intact surfaces were used for determination of major elements, carbonate, and fluoride; lesions showing cavitation were studied for trace elements. In the study of dentinal lesions all samples were obtained from soft carious dentin.

For the electron microscopic work whole teeth or specimens of sound and carious tissue were fixed immediately after operative removal in cold (4°C) 1 per cent osmic acid solution containing sucrose (0.22 M) and buffered to pH 7.2–7.4 with 0.025 M veronal acetate buffer; or in neutral 10 per cent formalin or in 70 per cent alcohol. After fixation some of the material was dehydrated in alcohol and embedded in butyl methacrylate catalyzed with 1 per cent benzoyl peroxide. Some material was stored in 70 per cent alcohol and sectioned without further treatment. A few observations were also made on fresh untreated tissues.

Observations on the inorganic phase of enamel and dentin were made on ultrathin sections cut with a Porter-Blum microtome equipped with a glass knife, and an LKB Ultratome equipped with a diamond knife. Samples of enamel gently triturated in distilled water were studied without embedding. The collagen-crystallite relationships in dentin were examined in tissue samples homogenized in distilled water employing a Dounce ground-glass homogenizer.

For study of the organic phase the tissues were usually demineralized prior to embedding. To protect the enamel matrix, the tissue samples were maintained within a dialysis bag during demineralization with EDTA (0.46 M, pH 7.2–7.4) (Burrows, 1961), and also during the subsequent washing, dehydration, and infiltration procedures. After embedding in butyl methacrylate and sectioning, the tissue was stained with a lead preparation (Watson, 1958). Dentin samples were demineralized in EDTA or in formic acid–sodium citrate decalcification solution (Morse, 1945) prior to embedding. Sections of dentin were stained with 10 per cent phosphotungstic acid solutions for periods from 10 minutes to 2 hours. Micrographs were taken with a Siemens Elmiskop Ia operated at 60 or 80 kv. (For details of procedures, see Johansen and Parks, 1961, 1962.)

The chemical studies were performed on samples of enamel and dentin prepared by mechanical separation of the tissues using diamond wheels, burs, or hand instruments. This procedure has been found to give enamel and dentin samples of high purity. In studies of the inorganic phase the organic matrix was eliminated by ashing at temperatures of 550 to 600°C. Dry weights of the samples were obtained by drying in partial vacuum for 24 hours at 105°C.

For specific elements and for carbonate determinations the following procedures were used. Calcium and magnesium combined were determined by complexometric titration with EDTA, using eriochrome black T as indicator (Biedermann and Schwarzenbach, 1948). Magnesium was then determined separately according to a colorimetric method (McCann, 1959). For phosphorus the colorimetric method of Chen *et al.* (1956) was employed. Carbonate determinations were performed on the standard VanSlyke manometric apparatus (VanSlyke and Folch, 1940), modified for solid samples (Sobel *et al.*, 1943) with solutions prepared according to MacFadyen (1942). In the determination of fluoride the traditional steam distillation was employed (Willard and Winter, 1933), and fluoride in the distillate was determined by spectrophotometric methods (Megregian, 1954). (Specifics of procedures will be published in detailed reports on these studies.)

Results

Ultrastructure

A conspicuous feature of many sections of carious enamel in an advanced stage of destruction was the occurrence of distinct structureless areas of varying width delimiting enamel rods (Fig. 1), and corresponding to rod sheaths in location (Figs. 4 and 11). Another characteristic was the sparse distribution of crystallites within the carious tissue (Figs. 1 and 2). These findings indicated extensive demineralization within rods and in rod sheath and interrod areas, resulting in spaces which appeared to form a system of microchannels in the tissue. The occurrence of such a system was readily demonstrated by immersing a carious lesion of enamel in silver nitrate solution. Within a few minutes the electron-dense silver was found

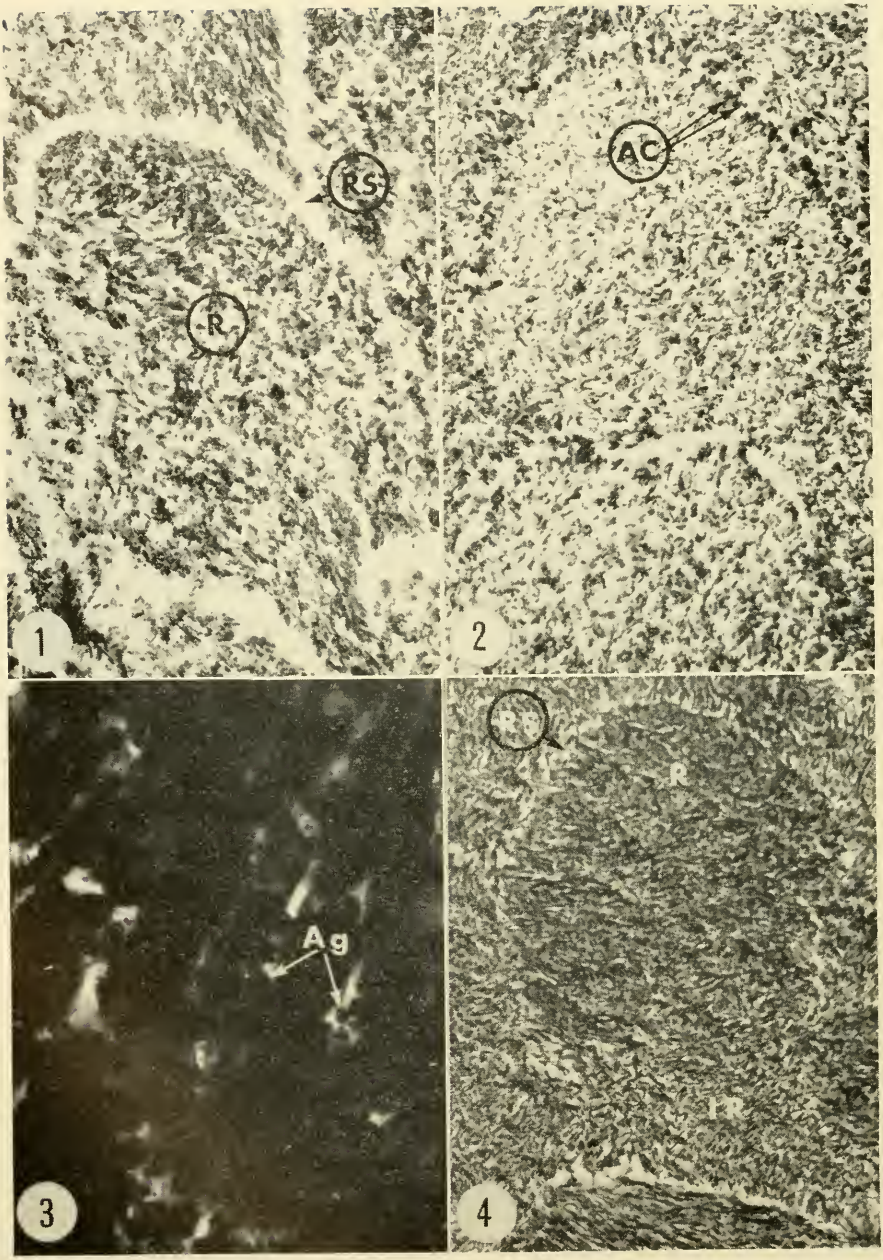
in spaces of enamel rods (Fig. 3). The sound tissue showed no evidence of penetration of silver in the same period of time.

Observations on the morphology, size, and electron density of crystallites within individual enamel rods at times suggested regional differences in these characteristics. The crystallites located at the periphery of rods appeared larger, more electron dense, and generally better preserved than those within rods (Fig. 2). It may be speculated that such crystallites have been selectively spared from dissolution or selectively recrystallized in the carious process.

Individual crystallites studied in sections and in triturated samples of carious enamel showed evidence of morphological changes (Figs. 5 and 6) referable to the carious process. The changes included localized and generalized surface erosion with establishment of spaces between adjoining crystallites (Figs. 6a and 6b). Also, perforations in transverse direction (Fig. 6c) were common findings. Occasionally, crystallites appeared to present perforations in longitudinal directions (Fig. 5). The most severely affected crystallites were seen as mere vestiges with little resemblance to the crystallites of sound enamel. Others (Fig. 6d) appeared remarkably similar to segments of crystallites observed in sound enamel (Figs. 7 and 8). The many apparent spaces seen in carious tissues suggested that many crystallites had been entirely dissolved.

Organic material was found in all samples of carious enamel studied after artificial demineralization, embedding, sectioning, and staining. Frequently, rod sheaths appeared distinctly outlined (Fig. 9), displaying a fine fibrillar component. In some preparations similar fibrils were also seen within rods and in interrod areas, while in other preparations these areas were devoid of matrix. (Interpretation of these findings in relation to the process of dental caries must, however, await further clarification of the modifying influence of certain technical procedures.) In other sections there appeared to be more organic material in the carious (Fig. 10) than in the sound tissue (Figs. 11 and 12). This finding may represent the presence of extraneous organic material derived from oral fluids. With the staining procedures available at present, however, it was not possible to distinguish between extraneous and intrinsic organic material.

Carious dentin studied in sections displayed a most interesting



FIGURES 1 TO 4

pattern of distribution of mineral. The greatest concentration was invariably found in areas lining the canals, often forming a hypermineralized pericanalicular layer interposed between the lumen of the canal and the extensively demineralized intercanalicular matrix (Figs. 13 and 14). It is not known whether this layer represents a persistence of the pericanalicular zone found in sound dentin (Fig. 15), or whether its occurrence is related to the carious process.

The most surprising finding in the study of carious dentin was the presence of distinct, relatively large crystallites in both pericanalicular and intercanalicular areas (Figs. 16 and 17). Stereoscopic observations (Johansen and Parks, 1960) showed that these structures resembled the crystallites of sound dentin in size and in their platelike shape (Figs. 18 and 19). This unexpected finding led us to propose that these crystallites persisted in the lesion because they were chemically different from the crystallites which had been dissolved. It was considered that fluoride preferentially deposited in the lining of dentinal canals and in persisting crystallites of intercanalicular areas could be responsible for this phenomenon (Johansen and Parks, 1959, 1961).

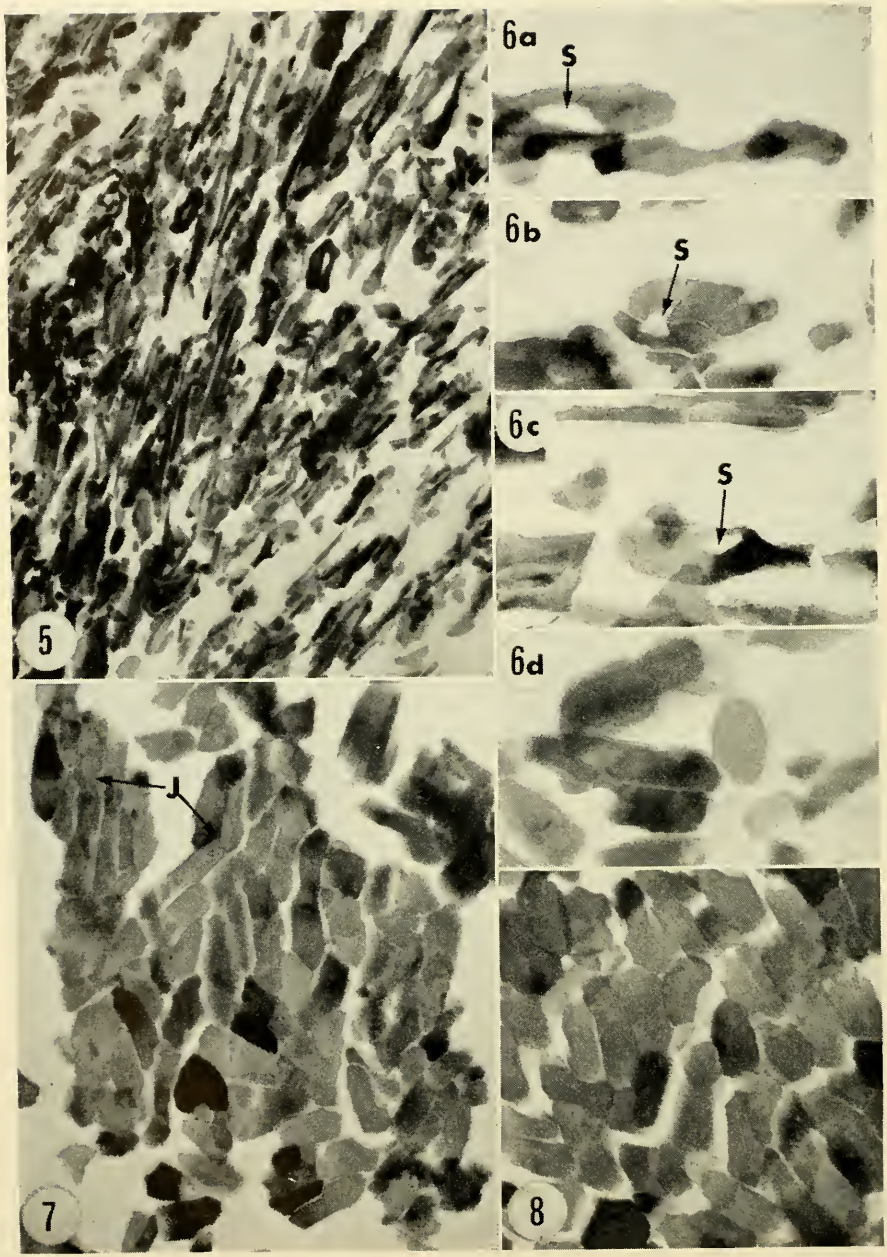
Crystallites of carious dentin were also found to be closely associated with collagenous fibrils isolated from homogenate preparations. Usually the crystallites were oriented with their long axes parallel to the long axis of the fibril (Fig. 20) in an arrangement similar to that seen in the heavily mineralized fibrils of sound dentin (Fig. 21) isolated by similar techniques.

FIG. 1. Section of undecalcified carious enamel showing enamel rods (*R*) outlined by wide rod sheath areas (*RS*). The sparse distribution of crystallites indicates extensive demineralization. (× approx. 14,250.)

FIG. 2. Section of undecalcified carious enamel. Crystallites (*AC*) at the periphery of rods are generally more conspicuous than those within rods. (× approx. 11,400.)

FIG. 3. Thin fragment of carious enamel exhibiting silver particles (*Ag*) within spaces in the tissue. The carious lesion was exposed to silver nitrate solution to test the permeability of the tissue. Dry preparation of specimen. (× approx. 66,500.)

FIG. 4. Section of developing deciduous human enamel, illustrating the general organizational pattern of the tissue. Rods (*R*) delimited by rod sheaths (*RS*) and separated by so-called interrod areas (*IR*) are seen. (× approx. 15,200.)



FIGURES 5 TO 8

Artificial demineralization of soft carious dentin and staining with phosphotungstic acid or lead preparations revealed an abundant matrix of collagenous fibrils (Fig. 22). The presence of typical cross-banding in longitudinal sections (Fig. 23) suggested that most fibrils were intact, resembling those of sound dentin (Fig. 26). However, areas with marked reduction in numbers of fibrils were seen, both in close proximity to bacteria (Fig. 24) and, in some regions, at a distance from bacteria. Observations on homogenate preparations of carious dentin also revealed collagenous fibrils with typical banding but devoid of crystallites (Fig. 25). The persistence of these fibrils in the carious tissue strongly supports the point of view that demineralization precedes proteolysis in dentinal caries. (For comparison with bone resorption see Hancox and Boothroyd, chapter 18 in this volume.)

Composition

Interpretation of the electron microscopic observations presented above raised a number of questions pertaining to the chemical composition of the carious tissues. In an attempt to clarify some of these points, a number of analyses were carried out. Several pertinent findings will be summarized by representative data.

Determination of major elements and carbonate of carious enamel revealed a reduction of calcium, phosphorus, magnesium, and car-

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Fig. 5. Section of carious enamel showing crystallites of varying size and shape. (× approx. 40,500.)

Fig. 6. Crystallites from carious enamel.

6a. Surface erosion with resulting space (S) between adjacent crystallites. Triturated preparation. (× approx. 77,000.)

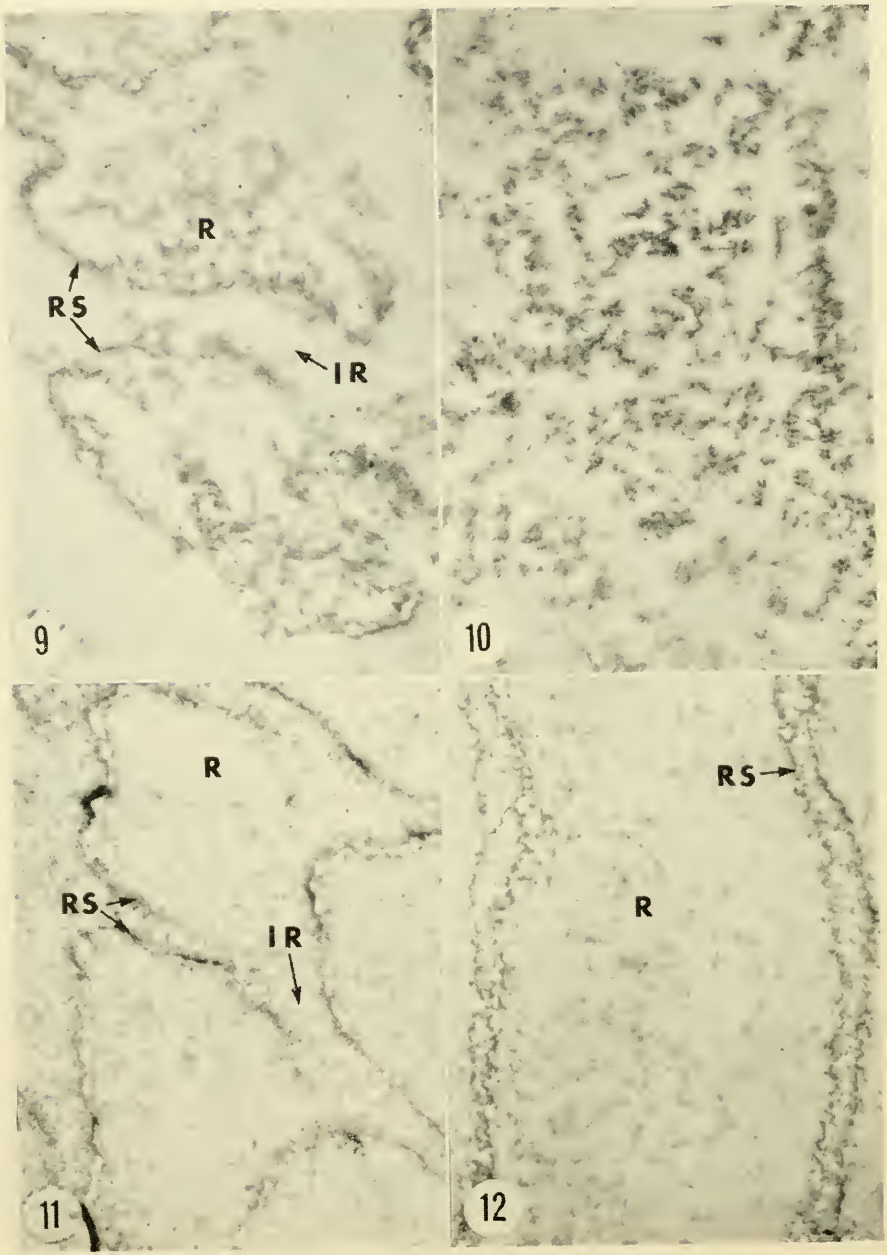
6b. Apparent localized erosion of adjacent crystallites with establishment of channel-like space (S). (× approx. 124,750.)

6c. Transverse perforation in crystallite (S). (× approx. 124,750.)

6d. Segments of well preserved crystallites within the lesion. (× approx. 124,750.)

Fig. 7. Section of sound permanent enamel showing distinct outlines of individual crystallites. Several apparent junctions (*J*) between ends of crystallites have been marked. (× approx. 103,700.)

Fig. 8. Section of sound permanent enamel illustrating obliquely cut crystallites. (× approx. 124,750.)



FIGURES 9 TO 12

TABLE I. MAJOR ELEMENTS, CARBONATE, AND WATER CONTENT OF SOUND AND CARIOUS HUMAN ENAMEL[a]

(Per cent dry weight; means of 20 samples. From Singh and Johansen, to be published)

Ca	P	Mg	CO_2	Ca/P	H_2O
Sound					
36.75 ± 0.17	17.41 ± 0.04	0.54 ± 0.01	2.42 ± 0.02	2.09 ± 0.02	2.02 ± 0.04
Carious					
35.95 ± 0.21	17.01 ± 0.06	0.40 ± 0.01	1.56 ± 0.03	2.08 ± 0.03	3.07 ± 0.05

[a] Lesions with intact surfaces (white spots).

bonate content (Table I). This was particularly noticeable on a volume basis, but the change was also statistically significant on a dry weight basis. The data show that the calcium/phosphorus ratio remained relatively unchanged in spite of the demineralization process. On a percentage basis the loss of carbonate and magnesium was much higher than that of calcium and phosphorus. This finding may indicate a preferential loss of magnesium and carbonate in the dissolution of crystallites, or it could possibly reflect a low concentration of these ions during the assumed recrystallization process.

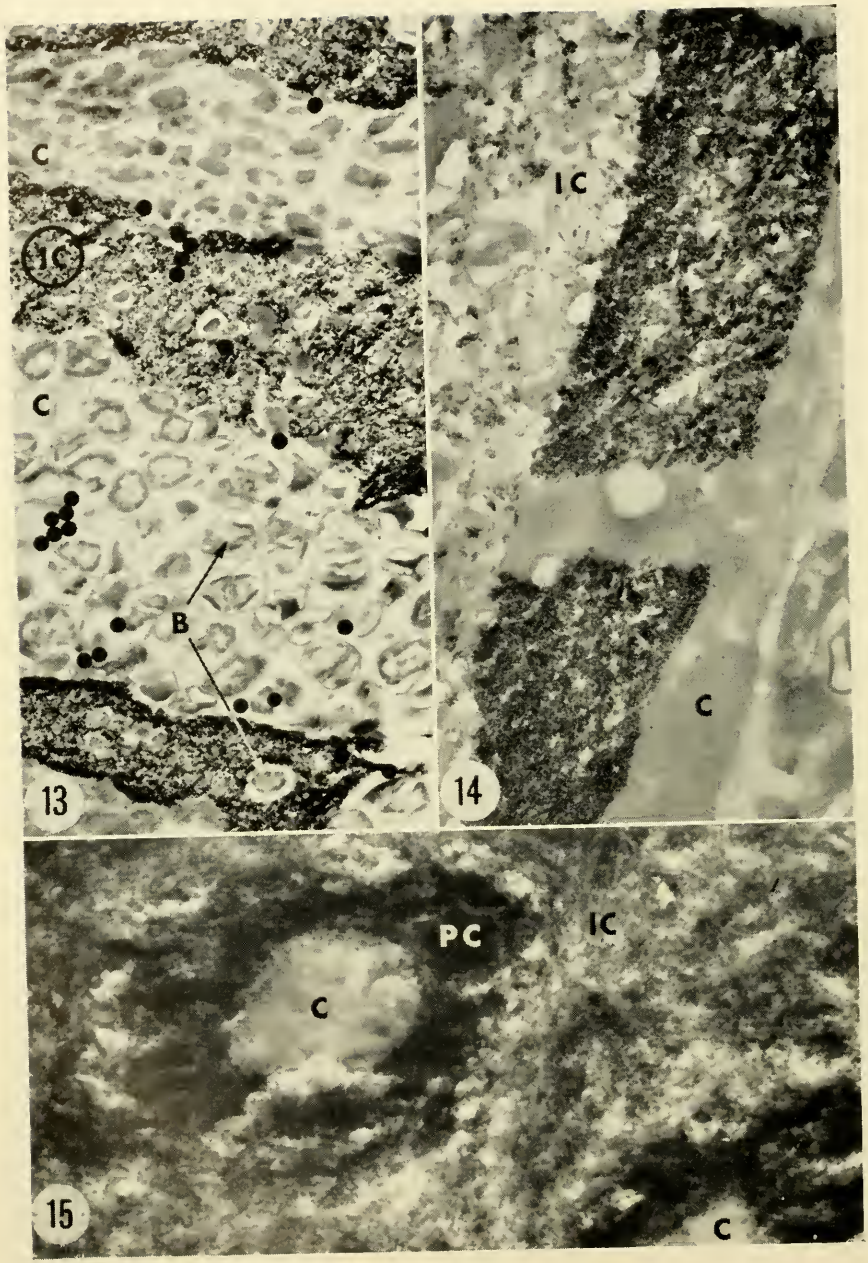
Observations on the fluoride content of carious enamel are found in Table II. In study I are presented fluoride values for total lesions compared with a corresponding sample of sound enamel. It can be seen that carious lesions on an ash basis contain 5 times more fluoride than the sound tissues. Since it is known that the fluoride concentra-

FIG. 9. Section of organic matrix from demineralized carious enamel stained with lead preparation. Rod sheaths (*RS*) are prominent in outline. Fine fibrils are frequently missing in interrod areas (*IR*) but are present within rods (*R*). (× approx. 10,500.)

FIG. 10. Section of organic matrix from demineralized carious enamel stained with lead preparation. An apparent increase in the amount of organic material is observed. (× approx. 35,650.)

FIG. 11. Section of organic matrix from demineralized sound enamel stained with lead preparation. The morphologic pattern of the tissue is readily recognized by rods (*R*), rod sheaths (*RS*), and so-called interrod areas (*IR*). (× approx. 8550.)

FIG. 12. Section of organic matrix from demineralized sound enamel stained with lead preparation. Longitudinally cut rods (*R*) delimited by rod sheaths (*RS*) exhibit a feltwork of minute fibrils. (× approx. 29,200.)



FIGURES 13 TO 15

TABLE II. The Fluoride Content of Sound and Carious Human Enamel[a]
(Ash weight. From Johansen and Nordback, 1962)

	Fluoride (ppm)	
	Study I[b]	Study II[c]
Carious	580	490
Sound: outer and inner layers	120	
Sound: outer layer		310
Sound: inner layer		90

[a] Lesions with intact surfaces (white spots).
[b] Pooled sample of 14 teeth. The sound sample was obtained by preparing cavities similar in size and shape to those resulting from removal of white spots.
[c] Pooled sample of 31 teeth. The sample labeled "outer layer" represents the sound enamel from the surface inward to one-half the depth of cavities resulting from removal of white spots. The "inner layer" represents the remaining enamel to the full depth of the cavity.

tion of surface enamel is higher than that of subsurface enamel (Brudevold *et al.*, 1956), it was also of interest to compare the fluoride content of the carious lesion with that of layers of sound enamel. The results of this comparison are presented in study II. Carious lesions again showed a higher concentration of fluoride than corresponding sound tissues.

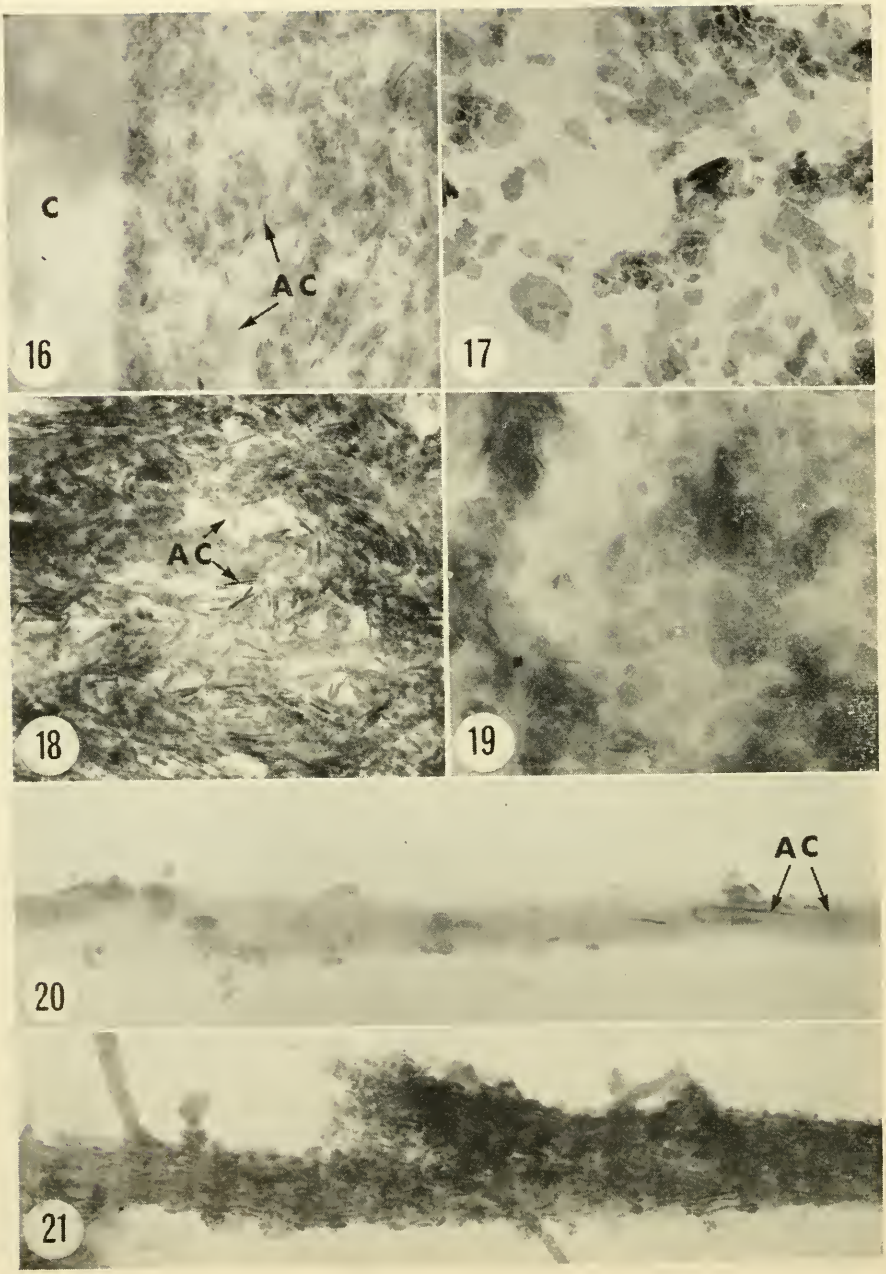
Some trace elements other than fluoride were also studied, and the results are presented in Table III. It was found that carious enamel showed a slight increase in lead, iron, and aluminum.

Studies on the composition of soft carious dentin on a weight basis

Fig. 13. Section of undecalcified soft carious dentin showing dentinal canals (*C*) filled with microorganisms (*B*). The intercanalicular matrix (*IC*) is largely but incompletely decalcified by the carious process and contains some scattered microorganisms (*B*) within lacunae. The polystyrene latex spherules (courtesy of Dow Chemical Company) are approximately 0.26 micron in diameter (× approx. 8000.)

Fig. 14. Undecalcified section of soft carious dentin showing a thick, densely calcified layer interposed between the lumen of the canal (*C*) and the extensively demineralized intercanalicular matrix (*IC*). (× approx. 26,000.)

Fig. 15. Section of embedded, undecalcified sound dentin. A zone of high mineralization (*PC*), approximately 0.5 micron wide, surrounds the lumen of the canals (*C*). The intercanalicular matrix (*IC*) shows an irregular but generally less dense distribution of mineral matter, occasionally displaying a banded appearance. (× approx. 15,000.)



FIGURES 16 TO 21

TABLE III. Trace Elements of Sound and Carious Human Enamel[a]
(Ash weight.[b] From Johansen, Steadman, and Steadman, to be published)

	Element (ppm)					
	Pb	Sn	Mn	Fe	Al	Sr
Sound	60	<3	<0.4	25	32	94
Carious	93	24	<1	52	93	99

[a] Soft carious enamel without surface layer; samples removed with spoon excavator.
[b] Pooled sample of 25 teeth.

revealed an increase in water and organic material and a decrease in ash (Table IV). Analysis of the ash fraction for major elements showed a marked decrease in magnesium with relatively minor changes in calcium and phosphorus contents. The calcium/phosphorus ratio was somewhat lower in the carious tissue than in sound dentin. On a dry weight basis, carious dentin also contained about 50 per cent less carbonate than sound dentin (Fig. 27), and the difference in rate of release at temperatures below 600°C might in-

Fig. 16. Section of undecalcified soft carious dentin embedded in methacrylate. Crystallites (*AC*) seen in edge view present thin, dark profiles; those seen in broad surface view are wider and less dense. At left is the lumen of a dentinal canal (*C*). (× approx. 74,650.)

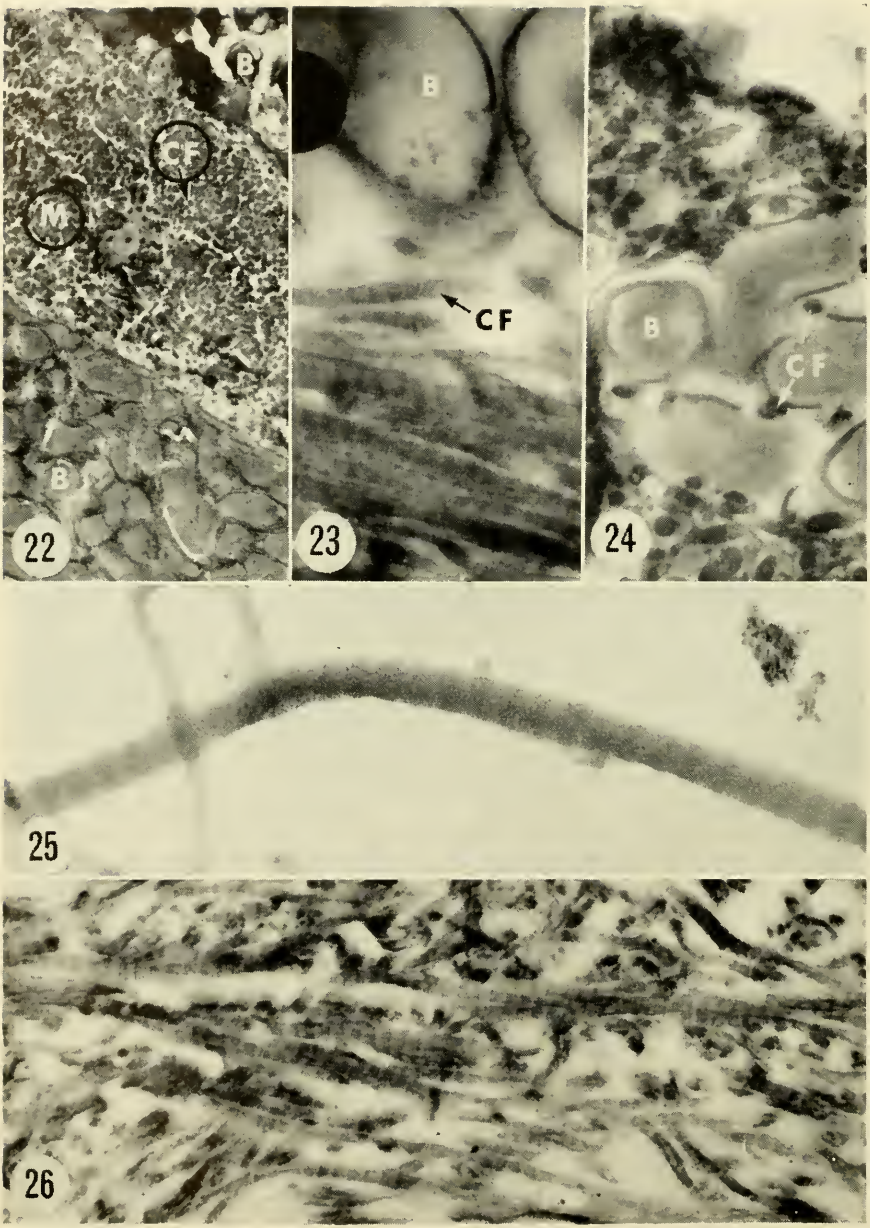
Fig. 17. Undecalcified soft carious dentin, showing the somewhat crumbled edge of a section taken from unembedded material. Most crystallites appear in broad surface view. (× approx. 96,800.)

Fig. 18. Section of undecalcified sound dentin embedded in methacrylate. Crystallites (*AC*) seen in edge view present thin, dark profiles; those seen in broad surface view are wider and less dense. (× approx. 74,650.)

Fig. 19. Undecalcified sound dentin, showing the somewhat crumbled edge of a section taken from unembedded material. In the thinnest parts, the crystallites appear in broad surface view; in the thicker parts they are seen in edge view. (× approx. 96,800.)

Fig. 20. Isolated collagenous fibril from homogenized preparation of carious dentin. A few crystallites (*AC*) in both broad surface view and narrow profile view are seen associated with the fibril. Very faint indication of banding is seen. (× approx. 69,600.)

Fig. 21. Isolated collagenous fibrils from homogenized preparation of sound dentin. The crystallites are oriented with their long axes parallel to the long axis of the fibrils. The alternating light and dark transverse bands are seen to be in register where the two fibrils join to form a single unit. (× approx. 70,000.)



FIGURES 22 TO 26

TABLE IV. Composition of Sound and Carious Human Dentin
(From Johansen and Nordback, 1962)

Tissue	Water	Organic[a]	Inorganic	⟶ Ca	P	Mg	CO_2	Ca/P
	Wet weight			*Ash weight*				
Sound	11.2	20.7	68.1	37.8 ± 0.83	17.75 ± 0.34	1.10 ± 0.18	2.20 ± 0.37	2.13 ± 0.04
Carious	58.0	27.4	14.6	38.4 ± 0.74	18.38 ± 0.42	0.27 ± 0.20	1.07 ± 0.36	2.08 ± 0.04
		Dry weight[b]						
Sound		23.0	77.0	27.5	13.0	0.83	3.50	
Carious		65.6	34.4	12.8	6.2	0.10	0.74	

[a] Organic and bound water.
[b] Inorganic composition corrected for CO_2 loss (see Fig. 27).

dicate differences in the location of carbonate in apatite crystallites from these two sources.

Of particular interest for the interpretation of the electron microscopic studies were the observations on the fluoride content of carious dentin. The data presented in Table V show that the inorganic phase of soft carious dentin contains about 10 times more fluoride than the corresponding sound tissue. The high fluoride content might thus be the most important factor in maintaining the crystallites within the carious lesion.

Studies on trace elements other than fluoride in carious dentin showed a fivefold increase in the zinc content (Table VI). Smaller

Fig. 22. Section of soft carious dentin decalcified and stained with phosphotungstic acid. The intercanalicular matrix (*M*) displays collagenous fibrils (*CF*) in transverse section. Dentinal canals are filled with microorganisms (*B*). (× approx. 10,450.)

Fig. 23. Section of soft carious dentin decalcified and stained with phosphotungstic acid. Collagenous fibrils (*CF*) with typical banding are found in close proximity to bacteria (*B*). (× approx. 53,700.)

Fig. 24. Section of soft carious dentin decalcified and stained with phosphotungstic acid. Numerous microorganisms (*B*) are seen within the intercanalicular matrix. A few collagenous fibrils (*CF*) persist between the microorganisms. (× approx. 37,300.)

Fig. 25. Isolated collagenous fibril from homogenate preparation of carious dentin, exhibiting banding and devoid of crystallites. (× approx. 68,500.)

Fig. 26. Section of intercanalicular matrix of sound dentin stained with phosphotungstic acid, showing a group of collagenous fibrils with distinct bands and interbands. (× approx. 31,100.)

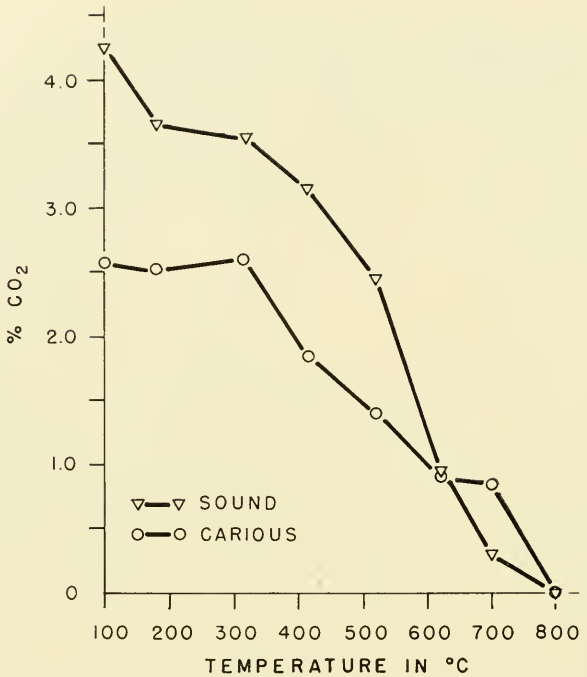


FIG. 27. The effect of heat treatment on the carbonate content of sound and carious human dentin.

increases in lead and iron were also noted. The relative significance of these elements in the carious process is not known.

DISCUSSION

A matter of particular interest in relation to the carious process was the pattern of distribution of mineral in carious enamel and dentin. It has been shown that dissolution of enamel may extend to a depth

TABLE V. FLUORIDE CONTENT OF SOUND AND SOFT CARIOUS HUMAN DENTIN
(Ash weight; means of 38 samples. From Johansen and Nordback, 1962)

	Fluoride (ppm)
Sound	180 ± 25
Carious	1730 ± 1400
Carious/sound ratio: 10.4 ± 7.0	

TABLE VI. Trace Elements of Sound and Soft Carious Human Dentin
(Ash weight; means of 12 samples. From Johansen, Steadman, and Steadman, to be published)

	Element (ppm)							
	Zn	Pb	Sn	Mn	Fe	Al	Ag	Sr
Sound	318	136	2	1.5	48	67	<0.4	120
Carious	1556	298	14	4	182	57	1.3	106

1000 microns beyond the location of the bacterial plaque on the tooth surface (Sognnaes, 1962). Such lesions frequently show an intact and densely mineralized surface layer, while deeper regions may be extensively but not uniformly demineralized (Gustafson, 1957). The electron microscopic findings show that apparent regional differences in degree of demineralization extend to the ultrastructural level. In enamel it was occasionally seen that crystallites at the periphery of rods were better preserved than the majority of crystallites within rods. Similar advanced demineralization of prism cores has also been noted in microradiographs (Darling, chapter 6 in this volume). The electron microscopic observations on dentin further showed more extensive demineralization in intercanalicular areas than in pericanalicular zones. On the basis of the latter observations it was hypothesized that the persisting crystallites in these regions were altered chemically and had become more resistant to dissolution (Johansen and Parks, 1959, 1961). This hypothesis is supported by the chemical data previously discussed (Johansen and Nordback, 1962) and summarized in this report.

It is known that the surface layer of enamel shows an increase in fluoride and a decrease in carbonate with age (Brudevold *et al.*, 1956; Little and Brudevold, 1958). This phenomenon may be part of a slow "maturation process" of dental tissues, not necessarily limited to the enamel surface. Deeper within the tooth, corresponding changes may occur on a limited scale in areas of relatively greater permeability. In enamel it is likely that rod sheath areas, because of their higher organic content, represent pathways of diffusion. In dentin, pericanalicular zones would appear to be more directly exposed to tissue fluid than would intercanalicular matrix.

By a slow exchange phenomenon, crystallites in regions of higher permeability might pick up fluoride from the surrounding fluid and possibly lose carbonate. Such a change in composition could account for the greater resistance to dissolution observed in crystallites in specific regions. Lack of such a "maturation process" in the area might be associated with a more uniform pattern of demineralization.

The marked increase in fluoride and the pronounced decrease in carbonate and magnesium observed in carious enamel and dentin, however, suggests more profound chemical changes than can be attributed to a maturation process, and an associated selective demineralization. The fact that many crystallites in all regions appeared well preserved lends support to the idea that these crystallites at least in part are products of a recrystallization process (Johansen and Parks, 1961). By such a mechanism the fluoride liberated from dissolved crystallites could be incorporated into those persisting; also, additional fluoride (Dowse and Jenkins, 1957) and other ions might be available from oral fluids for incorporation into the apatite lattice. The low magnesium and carbonate values, on the other hand, might reflect a low concentration of these ions in the fluid environment during recrystallization as well as preferential loss during demineralization. The recrystallization process would be dependent on changes in pH within the lesion and might involve an intermediate product of $CaHPO_4$ (Neuman and Neuman, 1958) as proposed by Gray and Francis (chapter 8 in this volume). The persistence of crystallites within advanced lesions might thus be explained on the basis of a reduction in solubility resulting from changes in composition.

The fate of the organic matrix in the carious process has long been a matter of controversy. The electron microscopic findings show that lesions in both enamel and dentin contain abundant organic material, and the chemical data confirm this observation. It has not, however, been possible to distinguish between extraneous and intrinsic organic material in lesions of enamel. In dentin, the matrix of collagenous fibrils appears relatively intact in areas of advanced demineralization, but its composition and susceptibility to break-

down by collagenase appear different from that of the sound tissue (Armstrong, 1961*a*).

In conclusion, it might be stated that the findings lend support to the general idea that demineralization precedes proteolysis in the development of carious lesions. It is evident, however, that the carious process in all its phases is a most complex phenomenon, at present not readily characterized in a single concept.

Summary

A series of electron microscopic and chemical studies were carried out to characterize carious lesions in enamel and dentin. The ultrastructural observations on the inorganic phase of the tissues were carried out on ultrathin sections, homogenate preparations, and triturated samples. After demineralization, the organic phase was studied in ultrathin sections stained with lead preparations and phosphotungstic acid, in unstained sections, and in homogenate preparations. The chemical composition was studied in samples that had been dried to constant weight or ashed.

In carious enamel, ultrastructural observations on the inorganic phase often revealed structureless areas of varying width delimiting enamel rods. Crystallites were found to be sparsely distributed, with some evidence for the existence of microchannels between them. Those at the periphery of enamel rods occasionally appeared to be in a better state of preservation. Individual crystallites were found to vary considerably in morphology; some showed generalized and localized surface erosion, some displayed perforations, and others appeared as mere vestiges with little resemblance to crystallites found in the sound tissue. Segments of seemingly unaltered crystallites were also encountered. Demineralized carious enamel studied in stained sections showed evidence of organic material. Persistence of the organic matrix was indicated by the occurrence of rod sheaths, even though rods and interrod areas frequently lacked the fine fibrils observed in the sound tissue. Sometimes an apparent increase in organic material was encountered. This finding was thought to be related to an influx of organic material from oral fluids.

The electron microscopic observations on soft carious dentin re-

vealed an interesting pattern of distribution of mineral. The highest concentration of crystallites was usually found adjacent to dentinal canals, whereas intercanalicular areas were extensively demineralized. Individual crystallites from both regions often appeared conspicuously large, indicating resistance to dissolution, possibly due to a change in their composition. Studies of the organic phase showed a remarkably intact collagenous matrix with individual fibrils displaying banding and cross striations. It was concluded that proteolysis follows demineralization in dentinal caries.

In the chemical studies of carious enamel and dentin it was found that the two tissues were in several respects similarly affected by the carious process. Both tissues showed an increase in water (and organic material) but a decrease in ash. Analyses of the ash fraction revealed relatively minor changes in calcium and phosphorus content, but a marked decrease in magnesium and carbonate. The fluoride content of carious enamel and dentin was much higher than that of the sound tissue. It was theorized that these chemical changes were, in part, brought about by a recrystallization process which resulted in the formation of crystallites of reduced solubility.

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8

Physical Chemistry of Enamel Dissolution

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INCIPIENT dental caries in enamel begins with the appearance of an opaque lesion, commonly known as a "white spot." The formation of this stage of caries is the result of chemical dissolution of enamel substance and, therefore, should be amenable to description in terms of physical-chemical principles. Such a description requires an identification of the factors affecting the process and a measurement of the magnitude of their effect on the process. The objective of this exposition is to present the physical and chemical factors affecting or controlling formation of an incipient carious lesion and to describe the process on a physical-chemical basis.

Recognition of the fundamentally chemical character of caries formation, beginning shortly before 1900 (Magitot, 1878; Miller, 1905), provided the inspiration for many investigations into the mechanism of this process. Concurrently with these investigations, a variety of systems, some of which included bacteria, were devised for producing the lesions *in vitro*, and, indeed, these methods were eminently successful. The essential process consists of the diffusion of the reactant to the reaction site, which in this case is the solid, enamel, followed by reaction and diffusion of the products away from the reaction site. Such an interaction between a solution and a solid is known as a heterogeneous reaction. In most cases, the rate of heterogeneous reactions is controlled by the rate of diffusion of

the reactant or solute to the solid surface. The diffusion rate is determined by the concentration of the reactant, and, therefore, the rate of reaction with the solid is directly proportional to the concentration of the reactant. Equilibrium solubility must also be considered because it may be attained frequently, and studies at equilibrium provide information which is difficult or impossible to obtain from rate data. These physical-chemical principles provide the basis for examining the formation of the carious lesion.

The following presentation can be regarded as a study of a model system for producing caries-like lesions irrespective of the chemical agents which cause caries. The primary reactant examined in this discussion is acid or acidic buffer. The model based on such an acid reactant is considered most promising because (1) adequate quantities of acid have been shown to exist in the immediate environment of the teeth, (2) an adequate substrate, hydroxyapatite, exists on which the acid can operate, and (3) caries-like lesions have been produced *in vitro* with acid.

The only other agents ever considered extensively as the primary reactants for enamel are proteolytic enzymes and complexing compounds. However, several attempts to produce a proteolytic enzyme effect in enamel resulting in caries-like lesions have not been successful (Burnett and Scherp, 1952; Jenkins, 1961). Furthermore, the rather small supply of substrate (i.e., organic content of enamel) upon which the proteolytic agents can act (Orban, 1957; Schatz *et al.*, 1956) makes this approach less logical. It does appear conceivable that compounds which can form strong soluble complexes with calcium, such as ethylenediaminetetraacetate, could be used to produce caries-like lesions in enamel (Ravnik and Löe, 1961), although no major attempts have been made to complete such a study. Many complexing agents have the necessary capability for dissolving the substrate, the inorganic portion of enamel, just as does acid. However, this concept becomes untenable *in vivo* because complexing agents of sufficient strength and in adequate concentration in the vicinity of the enamel surface (other than hydrogen ion) seem scarcely possible on the basis of a quantitative consideration of potential sources such as the organic moiety of enamel, bacteria, or

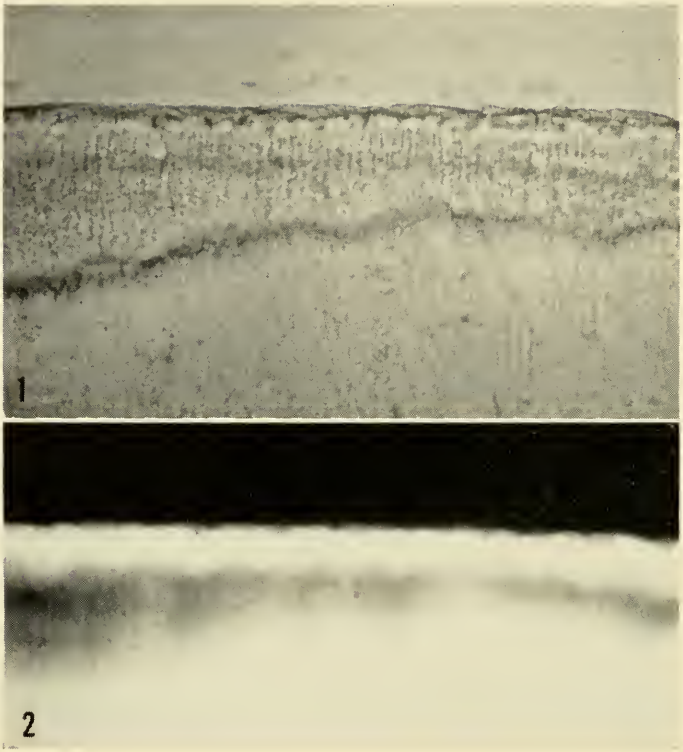
other organic constituents of plaque. A critique of available experimental evidence supports this view (Jenkins, 1961). On the other hand, hydrogen ion, in reality an extremely strong complexing agent for phosphate and hydroxyl groups, is adequately available. Should other agents be found important, however, they would also have to comply with the physical-chemical principles set forth earlier.

Morphology and Formation of Incipient Carious Lesions: A Brief Review

Carious Lesion Morphology

The morphology of an incipient carious lesion has been well described by Nishimura (1926), Darling (1956), Gustafson (1957), and Schmidt and Keil (1958), and is reviewed by Darling in chapter 6 of this volume. An example of an incipient carious lesion as described in these works is illustrated with photomicrographs (Figs. 1, 2, 3a, 3b, and 3c) taken of a section through a natural white spot. The typical early lesion consists first of an apparently sound layer of enamel at the surface. Beneath this surface layer is a region of decalcification where enamel substance has been removed in quantity. These regions can be observed by light microscopy (Fig. 1), but the proof of relative degrees of demineralization is provided by microradiography (Fig. 2). It is clearly apparent that there is a reduced mineral content in the decalcified subsurface region and a high mineral content in the relatively sound outer layer. Some loss of enamel substance from this relatively sound outer layer has been demonstrated by comparison with the outer layer of normal enamel, using precise microradiographic measurements (Soni and Brudevold, 1959).

The incipient carious lesion can be separated further into zones by observation with polarized light using a full-wave plate, first order red (Gustafson, 1957). A lesion showing these zones of birefringence (Fig. 3a, the same specimen as in Fig. 1) can be compared with the diagrammatic representation of Gustafson (Fig. 3b). In darkfield illumination, the lesion has a characteristic lighter appearance (Fig. 3c).



FIGS. 1 AND 2. A section through a typical natural incipient carious lesion with the original enamel surface retained, cut transversely from the mesial surface of an upper right first molar.

Fig. 1. Viewed with ordinary transmitted light, the lesion is apparent by the increase in contrast of the structural features as compared with normal enamel. (× 200.)

Fig. 2. A microradiograph of the lesion demonstrates the loss of material in the lesion that accentuates the interrod spaces. The relatively sound layer near the surface is readily apparent. (× 200.)

Therefore, an incipient carious lesion will be defined as a subsurface region of decalcification overlain by a relatively sound layer, with the surface completely intact, at least according to visual or light microscopic observations. It thus becomes apparent that the reactant must, for the most part, be by-passing the surface to diffuse into the enamel and react with subsurface enamel components.

Carious Lesion Formation

Incipient carious lesions of enamel formed in the oral cavity commonly occur under a bacterial plaque. The metabolic products of the bacteria that compose the plaque are almost certainly the destructive agents responsible for the attack on enamel. Unfortunately, definition and characterization of plaque is too incomplete to permit a chemical simulation of this system. In addition to establishing the composition and morphology of the entire plaque, which consists mostly of bacteria, it is necessary to define the composition of the free aqueous phase. Furthermore, knowledge of the interfaces existing at the exposed plaque surface and the enamel-plaque junction is needed for understanding the transport of the various chemical species from one site to the other. At the moment, one of the most deficient areas in caries research is this exact knowledge of the plaque system, which can at best be described as a complicated mixture of bacteria, enzymes, acids, other organic compounds, and inorganic ions such as calcium and phosphate. This enamel-plaque complex is the system of ultimate interest that needs to be described by rational, scientific principles as well as simulated in the laboratory.

The earliest attempts at producing carious lesions in the laboratory (Magitot, 1878; Miller, 1905; Pickerill, 1912) made use of mixtures of saliva with foodstuffs such as sugar or bread, and, in some cases, acidic solutions alone. These mixtures incubated with teeth for varying periods of time, but usually quite long, produced defects resembling both the incipient stage and the fully developed lesion with cavity formation. Such lesions covered the entire enamel surface unless the area of exposure had been limited by protective coatings. The importance of these experiments lay in the fact that caries-like lesions could be produced outside the oral cavity. Complicated as some of these systems were, they were more suitable for experimental study than the oral situation.

The next advances in the *in vitro* production of caries-like lesions followed two directions. One consisted in studying known bacterial cultures and nutrient systems, while the other involved elimination of bacteria to use only chemical systems. The classical example of the former is the "artificial mouth" devised by Pigman *et al.* (1952).

FIG. 3a. The section of the natural lesion of Fig. 1 as viewed with polarized light using a full-wave plate, first order red, has the typical appearance described and illustrated (Fig. 3b) by Gustafson (1957). All zones except the last highly decalcified fifth zone of an advanced lesion are visible. (× 200.)

FIG. 3b. A schematic representation of an incipient carious lesion mounted in Canada balsam, as viewed with polarized light using a full-wave plate, first order red, according to Gustafson, shows the five characteristic zones. (Reproduced from *Acta Odontol. Scand., 15,* Fig. X (1957), with the kind permission of the author and editor.)

FIG. 3c. The natural lesion of Fig. 1 as viewed in darkfield illumination with ordinary light. A lesion viewed this way appears lighter than the usual bluish gray of normal enamel and sometimes will almost glow as the surface does in this sample. However, the surface glow in this case was an edge reflection effect unrelated to the presence of the lesion. (× 200.)

FIG. 3d. A section of a lesion produced by a 96-hour exposure at 37°C to a medium consisting of 0.002 M $CaCl_2$ plus 6 per cent hydroxyethyl cellulose adjusted to pH 4.5 with hydrochloric acid, as viewed with polarized light using a full-wave plate, first order red. The similarity to the natural lesion of Fig. 3a and the diagram of Fig. 3b is apparent. (× 200.)

FIGS. 3e AND 3f. The same sections as in Figs. 8 and 9 and Figs. 12 and 13, showing the effect of increasing exposure time on lesions produced at 4°C with a medium of 0.05 M lactic acid and 0.03 M $CaCl_2$ plus 6 per cent hydroxyethyl cellulose adjusted to pH 3.5 when viewed with polarized light, full-wave plate, first order red.

Fig. 3e. After a 30-hour exposure only zone 1 of dark blue or purple described by Gustafson was visible. The sound outer layer was just appearing. (× 200.)

Fig. 3f. At 120 hours, zone 2 (gold band) had appeared. (× 200.)

FIG. 3g. A section of a lesion produced by a 96-hour exposure at 37°C to a medium consisting of 0.01 M lactic acid and 0.03 M $CaCl_2$ plus 6 per cent hydroxyethyl cellulose (with high buffer content) adjusted to pH 3.5. At this high rate of decalcification a whitish zone was formed in place of the usual gold appearance of zone 2. (× 200.)

FIG. 3h. A section of a lesion produced by interrupted exposures at 37°C to a medium consisting of 0.05 M lactic acid and 0.03 M $CaCl_2$ plus 6 per cent hydroxyethyl cellulose adjusted to pH 4.0. The sample was removed every 48 hours, washed 5 minutes with distilled water, and returned to the decalcification medium. The total exposure was 240 hours. Zone 2 (gold band) became very broad by reason of the long cycling exposure. (× 200.)

FIG. 3i. A section of enamel (same as in Fig. 37) after a 15-day exposure at room temperature to a solution of hydrochloric acid at pH 4.5, viewed with polarized light using a full-wave plate, first order red. A decalcification near the surface is visible and there are signs of zones similar to those seen in Figs. 3a and 3b. (× 200.)

FIG. 3j. A section of enamel (same as in Fig. 38) after a 15-day exposure at room temperature to an aqueous solution of 0.0005 M $CaCl_2$ and 0.0005 M NaH_2PO_4 adjusted to pH 4.5, viewed with polarized light using a full-wave plate, first order red. The appearance of the lesion bears no resemblance to Figs. 3a and 3b. The uniformity throughout the lesion is striking. (× 200.)

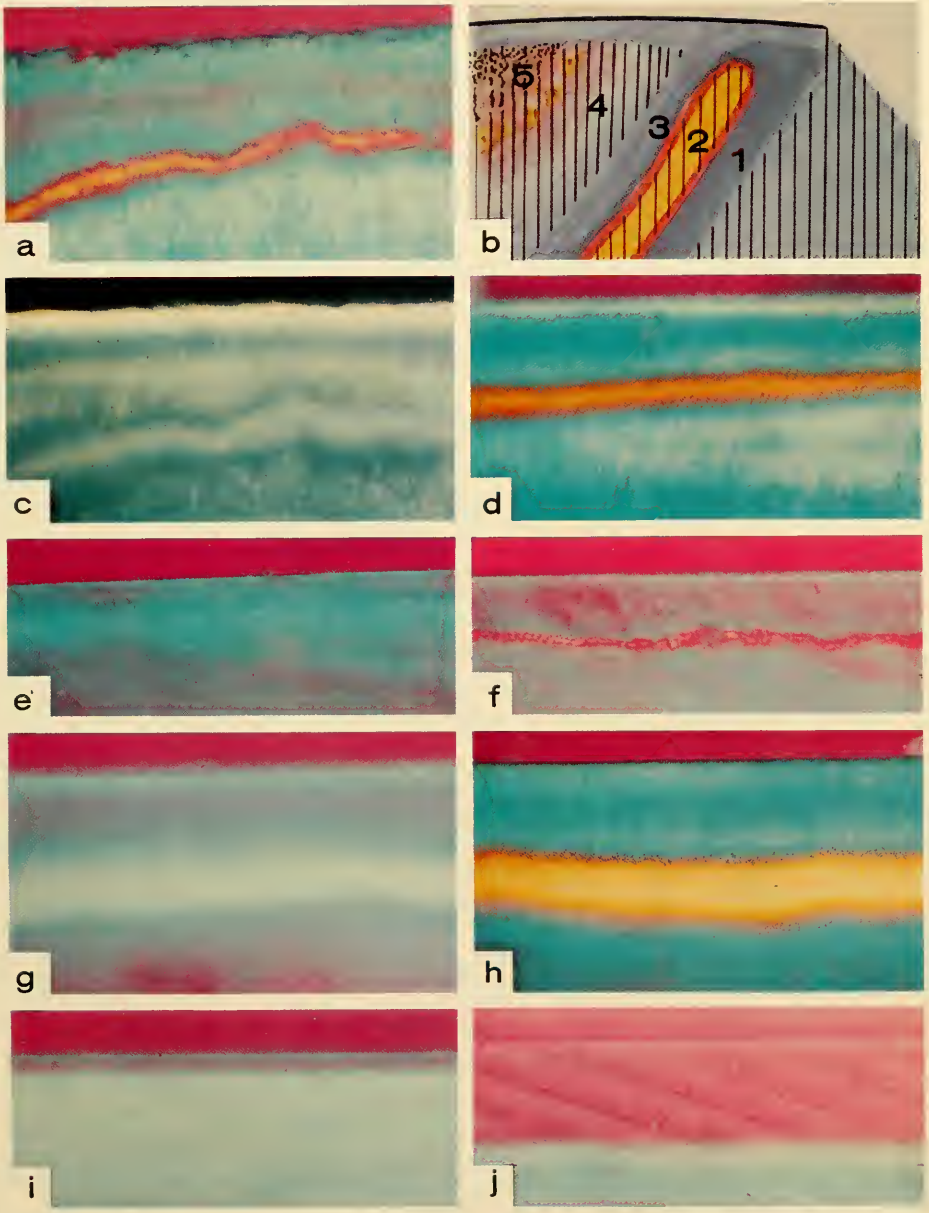


Figure 3

This system permitted the production of lesions in all the various stages and demonstrated the wide variety of defects obtainable with different bacterial strains. Several other methods have been developed for producing incipient caries-like lesions using a nutrient solution mixed with saliva or bacteria (Gore, 1940; Darling, 1956; Newbrun *et al.*, 1959; Soni and Bibby, 1961). Francis and Meckel (1963) devised such a method using an agar base containing the nutrient and saliva that produced lesions in a matter of days and was sufficiently well controlled for use as a routine test method. A bacterial medium inoculated with *Lactobacillus casei* and sterilized after a suitable incubation time was used to show that the products of bacterial metabolism, such as acids, were responsible for producing the caries-like lesions rather than the bacterial enzymes or the bacteria per se (Briner, 1963). Teeth that were immersed in such a medium developed the same defects as those found in a medium containing viable organisms. The production of such lesions in enamel has been achieved using only acid mixed with a viscous or gel base, omitting entirely both the organisms and the nutrients (von Bartheld, 1958; Mühlemann, 1960; Opdyke, 1962).

One of the more extensive studies of a chemical system for producing incipient caries-like lesions in enamel was done using acidic solutions containing calcium and phosphate (Coolidge *et al.*, 1955). A definite relationship among the hydrogen, calcium, and phosphate ion concentrations was found to be necessary in order to obtain characteristic incipient lesions. Similar observations were made using a novel technique whereby the progress of the lesion could be observed on a section of a tooth (Wachtel and Brown, 1963). A detailed study was also made of lesions produced by lactic acid alone (Hals *et al.*, 1955), and the various combinations of subsurface dissolution and surface dissolution were described. Another investigation was made using acetic acid alone, where the lesions were examined with polarized light and microradiography (Soni and Brudevold, 1960).

In a study of the dissolution of enamel in acidic buffers, the important factors affecting the reaction between acid and hydroxyapatite were isolated (Gray, 1962). The rate of dissolution was found to be primarily dependent on and proportional to the undis-

sociated acid concentration. The rate was also inhibited by the presence of cations and selected anions. Although this study dealt only with surface decalcification, and the white spots typical of incipient carious lesions were not produced, the relationships determined also apply to some stages of incipient carious lesion formation. On this basis, an elementary mechanism was proposed for incipient carious lesion formation (Gray *et al.*, 1962) founded on the diffusion of undissociated acid into enamel to cause subsurface decalcification. In addition, it was suggested that this system was influenced by the presence of an organic coating to protect the enamel surface, as will be discussed subsequently.

More recently, the rate of growth of incipient carious lesions was measured (Kapur *et al.*, 1962) using a technique similar to that of Wachtel and Brown (1963). The lesions were produced with aqueous solutions of either lactic acid or acetic acid, and the formation rate was determined by measuring the depth of the lesion as a function of exposure time. The rates of formation were then measured as a function of pH and buffer concentration. The significant conclusion reached in this study was that the rate of formation was proportional to the concentration of undissociated acid following, in essence, the mechanism proposed by Gray *et al.* (1962).

Study of Incipient Carious Lesion Formation Using Chemical Systems

Cardinal Factors Affecting Decalcification Systems

The study of the physical chemistry of incipient carious lesion formation required the development of a system that would imitate the *in vivo* circumstances and would be suitable for a quantitative evaluation of the variables of the system. Acid or acidic buffer constituted the fundamental element of the dissolution or decalcification medium. Lactic acid was chosen on the basis of its being one of the important acid products of bacterial metabolism. The organic coating protecting the enamel surface, mentioned previously, was considered essential, and a material to provide such a coating constituted another fundamental element of the decalcification medium.

The major reaction products—calcium and phosphate—also were incorporated into the decalcification medium at appropriate stages in the study. Carbonate, another reaction product, was not considered in this preliminary investigation, but may have to be accounted for in later, more sophisticated systems.

It is obvious that if a homogeneous solid sample of hydroxyapatite is exposed to acid, attack will begin at the surface. It is also obvious that if diffusion pathways within such a sample are available, the acid will tend to diffuse into those regions of the sample having lower acid concentrations. The presence of diffusion pathways in normal sound enamel has been amply demonstrated (Pickerill, 1912; Sullivan, 1954). Therefore, the logical expectation for a system consisting of enamel exposed to acid is that acid will diffuse both to the enamel surface and *into* the enamel. If the acid is consumed very rapidly at the surface, then little or no acid will remain to diffuse into the enamel. If, on the other hand, the surface reaction is retarded, more acid will diffuse farther into the enamel until subsurface reaction occurs. Thus, conceivably, there could be competition between (1) reaction at the surface and (2) diffusion into enamel with subsequent subsurface reaction. In the event that the rate of the first step could be greatly reduced relative to the second step, then the result would be an incipient caries-like lesion. The studies by Hals *et al.* (1955) support this idea of a competition between surface and subsurface reaction.

The wide variety of conditions encountered in the formation of incipient carious lesions *in vivo* and *in vitro* poses the problem of how the surface reaction can be uniformly retarded in all these systems. A review of both the natural and the artificial systems which have been reported indicates, as far as can be determined, that in every case organic material was present. This conclusion is based on the following considerations. In the oral cavity there are present the enamel cuticles, bacterial plaque, and saliva. *In vitro* systems using bacteria contain proteins, polysaccharides, mucoproteins, or mucopolysaccharides. The enamel surface as it occurs in the oral cavity is less soluble than subsurface enamel, and this property has been ascribed, in part at least, to the presence of certain inorganic

ions, such as fluoride (Brudevold, 1948). It has been shown, however, that a reduction in solubility approaching almost complete protection results from the naturally occurring organic coating on enamel, the acquired enamel cuticle (Meckel, 1961), and this deposit has since been found to penetrate enamel to a depth of several microns, which makes its removal difficult. In previous studies of chemical systems which produced caries-like lesions in enamel, the original enamel surface was left essentially intact, retaining either all or remnants of the insoluble organic coatings and the inorganic ions which reduce enamel solubility.

On the basis of the preceding argument, an organic material was considered to be a necessary component of any decalcification medium in addition to acid and acidic buffer. Furthermore, in order to define the role of the organic material to be used in making incipient carious lesions *in vitro*, the enamel should retain none of the original surface and should consist entirely of subsurface enamel. For this purpose, all samples of enamel used in the present studies were acid etched after mounting and ground with abrasive to remove the surface layer as ascertained by solubility rate measurements (Gray, 1962). Enamel samples consisted of squares (approximately 4×4 mm^2) cemented to the ends of a plastic rod with a dental plastic. All sides of the enamel were coated with the plastic cement, leaving only the surface exposed.

A suitable organic material was selected experimentally. An examination of past effective *in vitro* systems indicated that such a material should be polymeric. Meckel (personal communication) in studying the components of the agar-saliva system (Francis and Meckel, 1963) found agar, saliva, proteins, and selected amino acids to be effective to varying degrees. A variety of water-soluble polymers was then tested using synthetic chemicals, natural products, and derivatives of natural products in an attempt to find an effective, well characterized material. The test consisted of mixing the polymer with a solution of 0.05 M lactic acid, immersing a sample of enamel in a few grams of the medium, and examining the sample visually at intervals for defects resembling incipient carious lesions. Control areas were provided by coating a corner of the enamel with a dental cavity lining varnish. The qualitative results of such tests

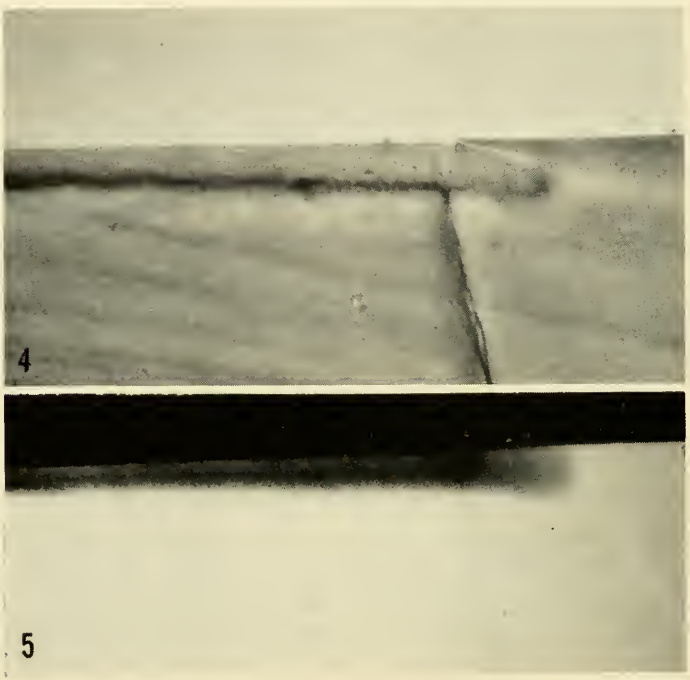
TABLE I. Effectiveness of Water-Soluble Polymers Mixed with Acidic Buffer for the Formation of Incipient Caries-like Lesions and Inhibition of Dissolution of Enamel Surface

Name	Effectiveness
Hydroxyethyl cellulose	Excellent
SeaKem (carrageenan, an acidic polysaccharide)	Good
Tomato juice agar	Good
Carboxymethyl cellulose	Fair
Carboxymethylhydroxyethyl cellulose	Fair
Polyvinylpyrrolidone	Fair
Methyl cellulose	Fair to poor
Polyox (ethylene oxide condensate)	Poor
Cab-o-sil (finely divided silica)	No effect
Glucose	No effect
Polyvinyl alcohol	No effect

with several materials are listed in Table I. Synthetic polymers of simple structure, such as ethylene oxide condensates or polyvinyl alcohol, as well as an inorganic thickening agent, finely divided silica, were completely ineffective. The solutions dissolved the enamel surface just as would have occurred if the polymer had been absent. The most effective compounds were polysaccharides, such as derivatives of cellulose. The testing of eligible materials was by no means exhaustive, but complexity, lack of purity, and undesirable ionic properties precluded interest in many undoubtedly effective compounds. Hydroxyethyl cellulose, a nonionic synthetic derivative of cellulose, was unusually effective and was selected as the polymeric ingredient of the decalcification medium. The choice of hydroxyethyl cellulose was based on (1) effectiveness, (2) nonionic character, (3) inertness to salt effects, (4) the fact that hydroxyethyl cellulose thickened rather than solidified the solutions and permitted a good contact between enamel and the decalcification medium, and (5) minimal changes in viscosity over the temperature range of interest. The particular samples used were found to contain an acidic buffer salt, sodium acetate, which had to be taken into account when total undissociated acid was calculated. The amount of this buffer capacity was determined from pH titration curves using both acid and base titrants.

Comparison of in Vitro and Natural Lesions

The equivalence of natural incipient carious lesions to those produced by the *in vitro* system will be established, and, at the same time, the effects of some of the variables will be qualitatively described. The microscopic appearance, using ordinary transmitted light (Fig. 4), of a lesion produced in 24 hours with a solution of



FIGS. 4 AND 5. A section through an incipient caries-like lesion produced by a 24-hour exposure at 4°C to a medium consisting of 0.05 M lactic acid plus 6 per cent hydroxyethyl cellulose adjusted to pH 3.5.

Fig. 4. In ordinary transmitted light, the lesion can be seen to have penetrated about 30 microns. The right side of the surface, protected from the medium by a varnish coating, provides a control for comparison. (× 200.)

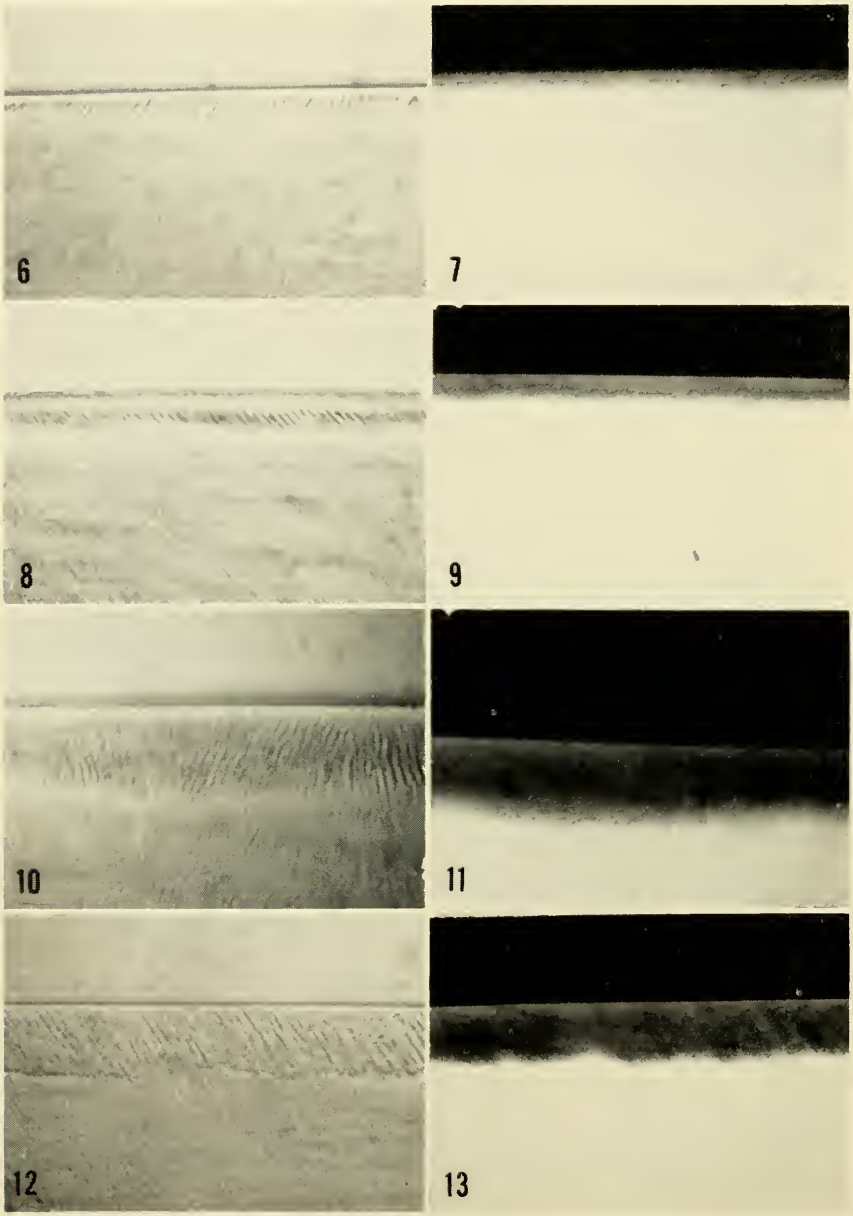
Fig. 5. A microradiograph shows that a considerable loss of material from the subsurface lesion had occurred. There is slight evidence of a relatively sound outer layer at the surface over the decalcified region of the lesion. The surface of this sample, when viewed under a metallurgical microscope with top illumination, was slightly dissolved by the medium. (× 200.)

hydroxyethyl cellulose and lactic acid adjusted to pH 3.5, along with a microradiograph (Fig. 5) of the same section of enamel, illustrates the type of result obtainable. Lesions produced by this system definitely had a more sound layer, albeit not very deep, overlying the subsurface decalcified region. The surface of the enamel sample was only slightly changed, as determined by microscopic observation using a metallurgical microscope with top illumination.

The depth of the relatively sound outer layer at the surface can be enhanced by several devices: (1) increasing the exposure to the decalcification medium, (2) removing and returning the enamel sample to the decalcification medium at intervals, (3) adding calcium ions initially in the decalcification medium, (4) adding both calcium and phosphate ions initially in the decalcification medium, (5) increasing the buffer concentration, and (6) including one of many select cations, such as zinc, in the decalcification medium.

Examples of the lesion that resulted from adding calcium as $CaCl_2$ to the decalcification medium are presented in Figs. 6 to 13 for exposures of 16, 30, 90, and 120 hours. Figures 14 and 15 illustrate the effect of including both calcium (as $CaCl_2$) and phosphate (as NaH_2PO_4) to the medium (all at pH 3.5). There was a progressive increase in the depth of the relatively sound outer layer and in the loss of material in the decalcified region with each successive addition to the decalcification medium. Also, the enamel surface was now unchanged as determined microscopically. Adding phosphate (as NaH_2PO_4) alone caused severe surface damage. An even more dramatic improvement was found by doubling the lactate buffer concentration (Figs. 16 and 17) or by interrupting the exposure periodically (Figs. 18 and 19). The similarity of these lesions to natural lesions can be seen by comparison with Figs. 1 and 2. Adding zinc as $ZnCl_2$ had the same qualitative effect as $CaCl_2$, although higher concentrations of $ZnCl_2$ were required for quantitative equivalency.

The various zones observed in natural lesions using polarized light with a full-wave plate, first order red, have been well described previously (Gustafson, 1957), and a diagrammatic representation is reproduced (Fig. 3b). A chemically produced lesion with all zones



FIGURES 6 TO 13

except number 5 is shown in Fig. 3d. Zone 5, which represents the almost completely destroyed enamel, is beyond the scope of this study, but can be obtained by carrying out sufficiently long exposures.

The progression of the lesion began with zone 1 or zone 3 (dark blue or purple band near the surface, Fig. 3e), these zones being indistinguishable from each other. The relatively sound outer layer was formed next. This was followed by formation of zone 2 (gold band), which then separated zones 1 and 3 (Fig. 3f). Zone 4 (light blue band above zone 2) developed as the lesion progressed, followed by zone 5.

Although a detailed study of the formation of these zones as a function of the many variables of the decalcification was not made, a few relationships were discovered. The most significant was that at very high decalcification rates the yellow or gold birefringence colors of zone 2 became almost white with overtones of yellow, green, or blue (Fig. 3g). As the rate of decalcification decreased, hints of yellow and finally the familiar gold color appeared. At high decalcification rates, zone 2 was usually very narrow (Fig. 3f). Periodic interruption of the decalcification tended to broaden zone

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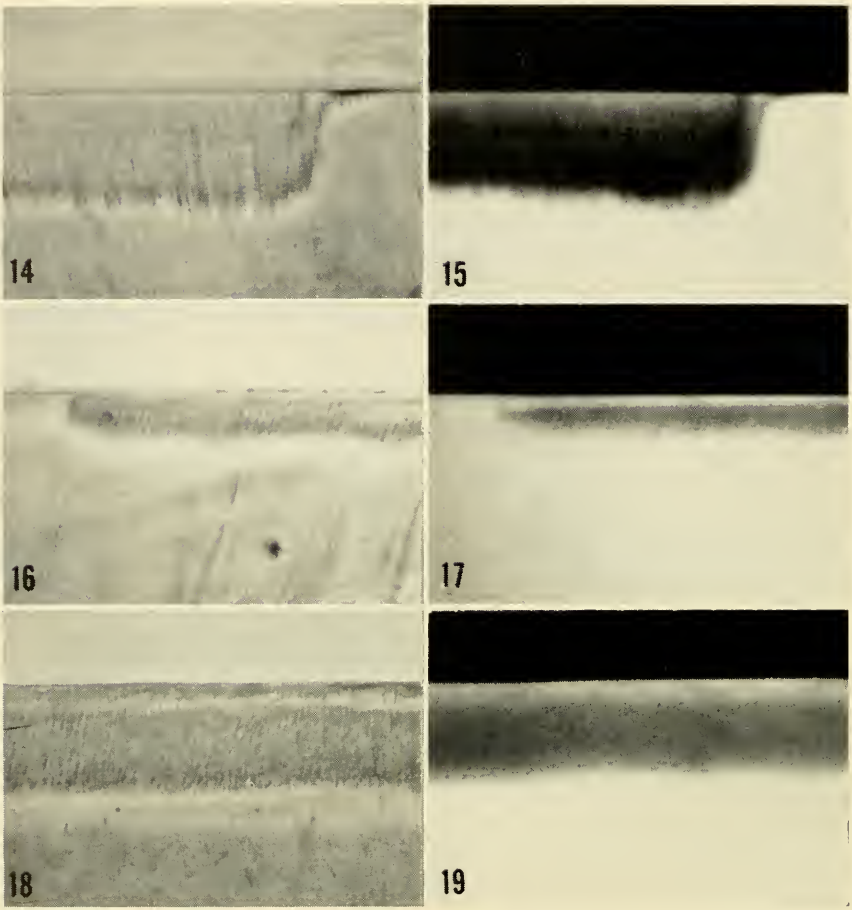
FIGS. 6 TO 13. The effect of increasing exposure time on the incipient carious lesion formed by exposure of enamel to a medium at 4°C consisting of 0.05 M lactic acid and 0.03 M $CaCl_2$ plus 6 per cent hydroxyethyl cellulose adjusted to pH 3.5 is demonstrated with light micrographs (Figs. 6, 8, 10, and 12) and microradiographs (Figs. 7, 9, 11, and 13) of sections from enamel samples exposed to the medium for different time periods.

Figs. 6 and 7. After a 16-hour exposure, decalcification at or near the surface, extending about 10 microns deep, was visible. (× 200.)

Figs. 8 and 9. After a 30-hour exposure, the lesion had progressed to about 25 microns and the relatively sound outer layer had appeared. (× 200.)

Figs. 10 and 11. After 90 hours, the lesion had penetrated about 50 microns and the relatively sound outer layer was about 5 microns wide. (× 200.)

Figs. 12 and 13. After 120 hours, there was a further increase in loss of subsurface enamel material and an enhancement of the relatively sound outer layer. About 50 per cent of the enamel had been dissolved from the lesion, according to calculations from the measured dissolved phosphate shown in Fig. 26. The surfaces of all the samples were almost untouched by the decalcification medium as determined microscopically. The depth of the lesion across each sample was also quite uniform. (× 200.)



FIGS. 14 AND 15. A section of an incipient carious lesion produced by a 96-hour exposure at 4°C to a medium consisting of 0.04 M lactic acid, 0.03 M $CaCl_2$, and 0.01 M NaH_2PO_4 plus 6 per cent hydroxyethyl cellulose adjusted to pH 3.5. The right side of the enamel surface was protected from the medium by a varnish coating.

Fig. 14. The light micrograph, taken with ordinary light, shows a lesion about 75 microns in depth and containing three bands. (× 200.)

Fig. 15. The microradiograph shows both the very great loss of subsurface enamel and the very considerable relatively sound outer layer. The surface of the enamel was unchanged as determined microscopically with top illumination. The increase in decalcification as compared with Figs. 10 and 11

[*Continued at bottom of following page*]

2 (Fig. 3h). Thus the decalcification mediums not only reproduce the zone differences of a natural incipient carious lesion, but also offer a means of studying these peculiar and little-understood changes.

An additional comparison of the natural and *in vitro* lesions was made by examination with electron microscopy (Figs. 20, 21, and 22). Diamond knife sections cut from undemineralized samples were used (Neal and Murphy, in preparation). Loss of substance from the interrod region was clearly apparent in both the natural (Fig. 20) and the chemically produced lesions (Figs. 21 and 22). The higher content of matter in the relatively sound outer layer was also demonstrable, as were the open pathways between the rods in this region. The advancing front of the lesion deeper in the enamel could be seen as an enlargement of the space between the rods that gradually disappeared in the normal enamel, while the body of the lesion consisted of large spaces with only scattered remains of the original enamel structure. The attack could be seen as beginning at

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resulted from the increase in acidic buffer capacity of phosphate, although the presence of phosphate further reduced attack on the enamel surface. (× 200.)

FIGS. 16 AND 17. A section of an incipient carious lesion produced by a 24-hour exposure at 4°C to a medium consisting of 0.10 M lactic acid and 0.03 M $CaCl_2$ plus 6 per cent hydroxyethyl cellulose can be compared with Figs. 8 and 9 to see the effect of increasing lactate buffer concentration.

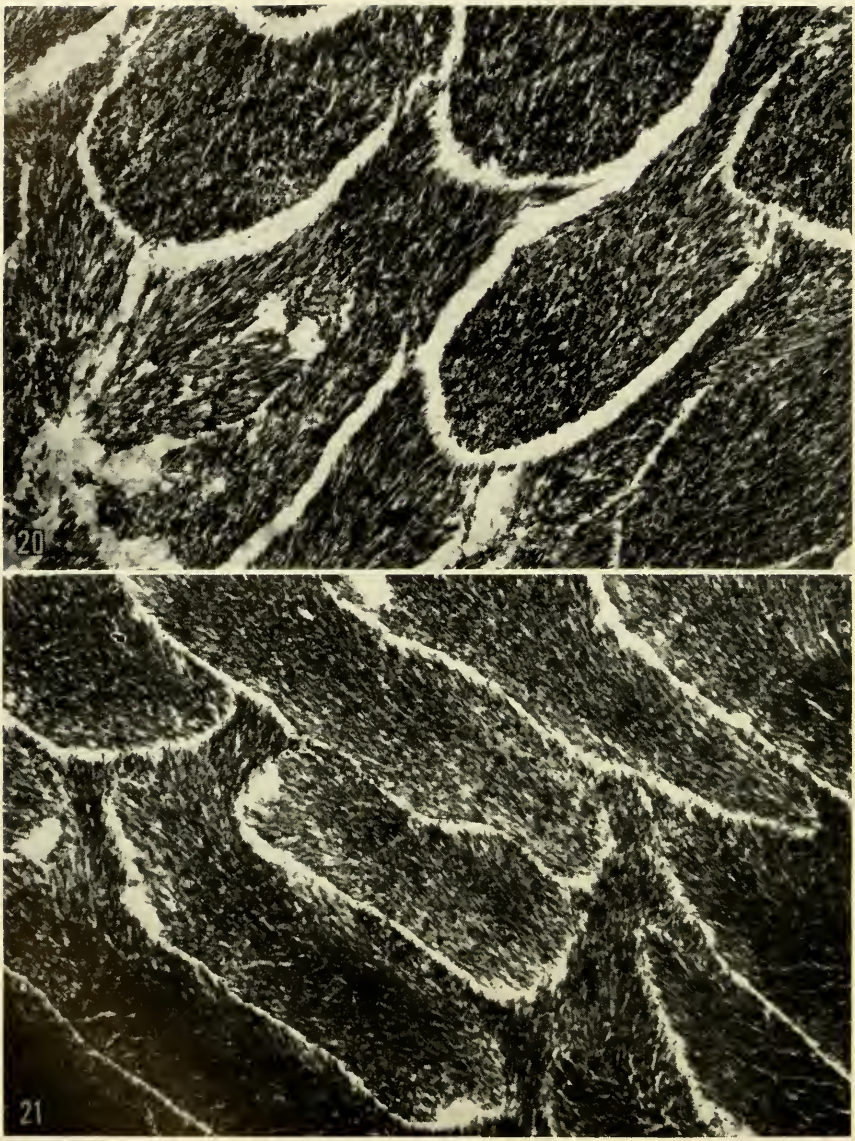
Fig. 16. In the light micrograph a very sharply defined relatively sound outer layer can be seen above the body of the lesion. (× 200.)

Fig. 17. The microradiograph confirms the presence of the relatively sound outer layer and the loss of subsurface mineral. (× 200.)

FIGS. 18 AND 19. A section of an incipient carious lesion produced by five 24-hour exposures at 4°C to fresh samples of a medium consisting of 0.05 M lactic acid and 0.03 M $CaCl_2$ plus 6 per cent hydroxyethyl cellulose adjusted to pH 3.5.

Fig. 18. A light micrograph shows the depth of the lesion and some bands within the lesion. (× 200.)

Fig. 19. A microradiograph proves the presence of a very wide relatively sound outer layer, amounting to 20 microns, and the loss of material from deep within the enamel. Interrupting or cycling the attack led to an increase in the sound outer layer, deeper penetration of the lesion, and slower decalcification of the body of the lesion. (× 200.)



FIGURES 20 AND 21

the outside of the rods and slowly permeating to the center of the rod.

Diamond knife sections of these samples with the embedding material (methyl methacrylate) removed were then demineralized with hydrochloric acid (0.005 N) to remove the inorganic substance and expose the organic content (Figs. 23 and 24). Normal mature enamel (Fig. 23) had a sparse organic residue which generally collapsed during the demineralizing procedure, so that little or no correlation with the original enamel structure was possible. In the incipient carious lesion region, produced either naturally or *in vitro* (*in vitro*, Fig. 24), the content of organic residue appeared to be much more appreciable and was easily correlated with the enamel rod structure, similar to enamel matrix of unerupted teeth, as well as the original undemineralized specimen (Fig. 21). The higher than normal organic content of natural lesions has been demonstrated by other investigators using chemical analyses and light microscopy (Bhussry, 1958; Johansen, 1962). The organic residue found in the present study seems to be concentrated in the enamel rod proper, where the greatest mineral content was observed. Furthermore, the highest organic content was always found in the relatively sound outer layer of the lesion at the surface and appeared to be located in the same vicinity as the mineral. Additional studies are in progress to identify the source of these residues and their relationship to the organic moiety of normal enamel.

The reality of the spaces observed in the incipient carious lesion by electron microscopy (Figs. 20, 21, and 22) can be demonstrated by infiltrating the enamel with a material which is visible in electron

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FIG. 20. An electron micrograph of a section through a natural incipient carious lesion cut from an undemineralized sample of adult human enamel using a diamond knife. The interrod spaces have been enlarged by the decay process and material has been leached out of the rod proper. (× 5000.) The appearance of normal enamel can be seen in parts of Fig. 22 or 36.

FIG. 21. An electron micrograph of a section through an incipient carious lesion produced by a 72-hour exposure at 37°C to a medium consisting of 0.05 M lactic acid and 0.03 M $CaCl_2$ plus 6 per cent hydroxyethyl cellulose at pH 3.5. The appearance is the same as that found in the natural lesion of Fig. 20, with loss of mineral from the interrod region and some leaching out of the rod interior. Undemineralized. (× 5000.)

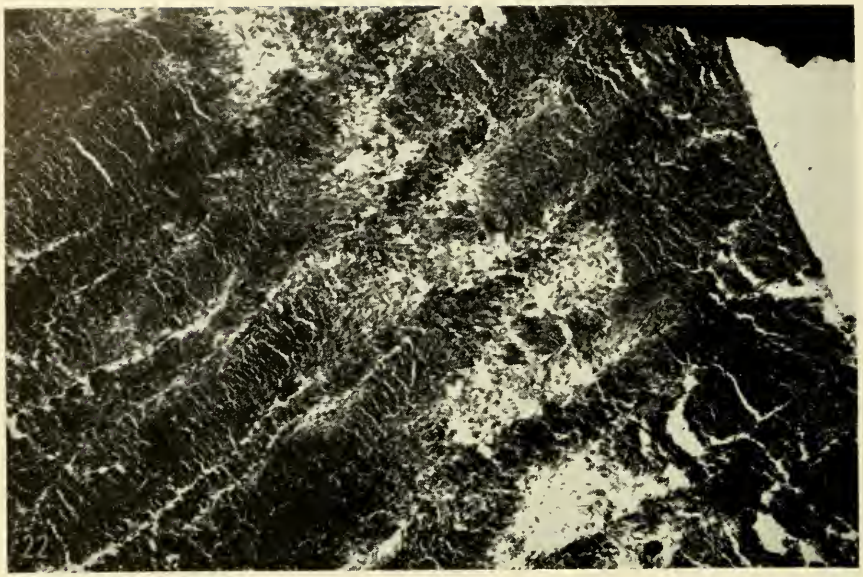


FIG. 22. An electron micrograph at low magnification showing an over-all view of an undemineralized section of a lesion produced by a 24-hour exposure at 4°C to a medium consisting of 0.10 M lactic acid and 0.03 M $CaCl_2$ plus 6 per cent hydroxyethyl cellulose adjusted to pH 3.5, from the same sample as Figs. 16 and 17. The enamel surface is in the upper right corner. The large areas containing no enamel coincide with the subsurface decalcified region seen in the microradiograph of Fig. 17. The relatively sound outer layer is still heavily mineralized, but some channels through this region following the interrod spaces are visible. The decalcification front approaching normal enamel is on the left side and was very obviously moving along between the rods. Sections of normal enamel have little or no space between the rods (upper left-hand corner of this figure or lower right-hand corner of Fig. 36). (× 2000.)

micrographs. The embedding material, methyl methacrylate, proved suitable for this purpose. It was found that the methacrylate penetrated to the very depths of the lesion and filled extremely small voids. The location of the methacrylate can be better visualized by demineralizing the sections with hydrochloric acid. Not only were extensive voids created in enamel during incipient carious lesion formation, but these voids were readily available to diffusing materials.

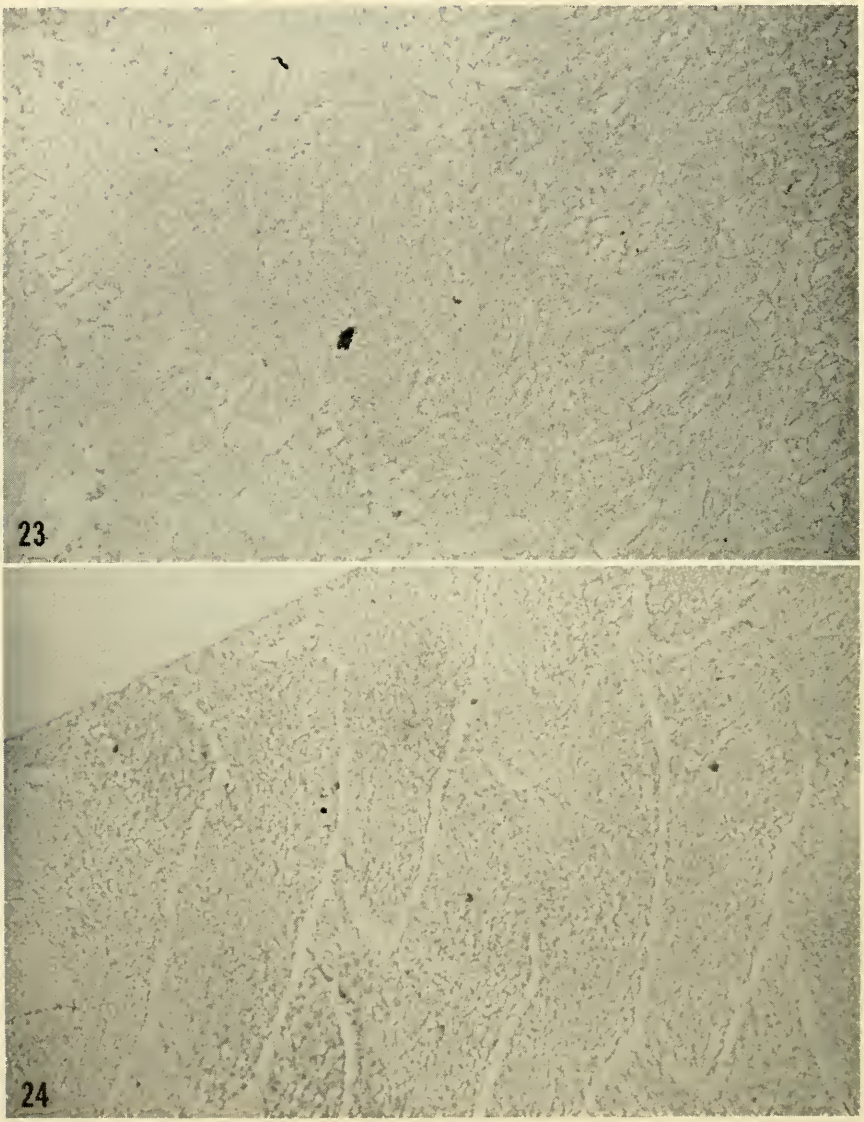
The lesions made *in vitro* advanced between the rods, along the striae of Retzius, and across prism cross striations. By all present

methods of observation, including histochemistry (Opdyke, 1962), the incipient carious lesion produced by the *in vitro* system is identical in appearance with natural lesions.

Variables Affecting Rate of Carious Lesion Formation

The rate of formation of the lesion produced *in vitro* was studied by quantitative measurements of calcium and phosphate released from enamel as a function of pH, buffer concentration, calcium concentration, and temperature.

The dissolution of enamel during incipient carious lesion formation was measured as a function of exposure time. Both an integral method and a differential method for determination of reaction rates were used (Laidler, 1950). The amount of enamel dissolved during exposure to the various mediums was determined by analyzing chemically for calcium (Welcher, 1958) and phosphate (Lucena-Conde and Prat, 1957). The calcium and phosphate analytical results were converted to weight of enamel, using its reported composition (Orban, 1957), and rechecked by analyzing known weights of dissolved enamel. By way of an example, the first medium to be discussed contained 0.05 M lactic acid, and hydroxyethyl cellulose was added in an amount of 6 gm per 100 ml of the lactic acid solution (hereafter referred to as 0.05 M lactic acid plus 6 per cent hydroxyethyl cellulose). The initial pH was adjusted with concentrated sodium hydroxide or hydrochloric acid as required. The amounts of enamel dissolved, determined by both phosphate and calcium analyses of the same solution (Fig. 25), were in good agreement, indicating no appreciable preferential dissolution of one component over the other during incipient carious lesion formation. This correspondence held during all subsequent measurements, and only the results of the phosphate analyses are presented in the figures to follow. The coefficient of variation in the determination of dissolved enamel was ± 11 per cent based on phosphate analysis and ± 13 per cent based on calcium analysis across different enamel samples and experimental conditions. The enamel samples used in these studies were examined for surface damage with a metallurgical microscope using top illumination, and then were sectioned with a special dental saw (Gray and Opdyke, 1962) for observation by



FIGURES 23 AND 24

microradiography and light microscopy and as a preliminary step in sample preparation for electron microscopy. The thickness of the sections varied between 50 and 100 microns. The sections were mounted on glass slides with Permount after microradiographs were taken.

The rate of enamel dissolution during incipient carious lesion formation followed a typical solubility curve (Figs. 25, 26, and 27). In every case, the rate of dissolution decreased with increasing length of exposure time, and the curves appeared to be approaching equilibrium. The decrease was not due to saturation of the bulk solution in all cases, as identical rates were found when the volume of the decalcification medium was increased tenfold. It is apparent that the dissolution rate is limited by changes in the composition of the medium in the vicinity of the enamel because of lack of time for equilibration with the bulk solution.

Adding calcium chloride to a medium containing 0.05 M lactic acid plus 6 per cent hydroxyethyl cellulose at pH 3.5 (Fig. 26) retarded the rate (Fig. 27). As pointed out previously, however, this addition caused a better-defined "relatively sound outer layer." Increasing pH of this same medium at a constant buffer strength caused a marked reduction in the rate of enamel dissolution (Fig. 27). Increasing temperature from 4° to 37°C had almost no effect (Figs. 25 and 26).

Further quantitative evaluation of the different variables was done by a differential method measuring the rate of dissolution at only

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FIG. 23. An electron micrograph of a section of normal enamel cut with a diamond knife and then demineralized with 0.005 N hydrochloric acid. The residue, which appears to be organic, is believed to be the remains of the organic matrix of enamel, but little of the original enamel rod pattern is recognizable. (× 5000.)

FIG. 24. An electron micrograph of a section cut from the sample of Fig. 21 and demineralized with 0.005 N hydrochloric acid. The incipient carious lesion was produced by a 72-hour exposure at 37°C to a medium consisting of 0.05 M lactic acid and 0.03 M $CaCl_2$ plus 6 per cent hydroxyethyl cellulose at pH 3.5. The enamel rod pattern is very apparent and resembles the picture in Fig. 21. The organic residue is located primarily in the rods and is more highly concentrated than in normal enamel. The highest concentration is always found at the surface (upper left-hand corner) in the relatively sound outer layer. (× 5000.)

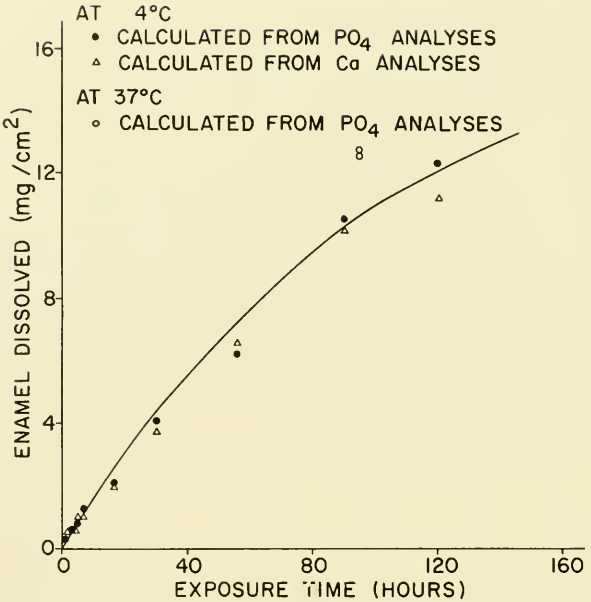


Fig. 25. Enamel dissolution as a function of time during exposure to a medium consisting of 0.05 M lactic acid plus 6 per cent hydroxyethyl cellulose (with low buffer content) adjusted to pH 3.5. The amounts of enamel dissolved as determined by either calcium or phosphate analysis agreed well. Temperature had little effect at 90 hours exposure. The enamel dissolution rate during incipient carious lesion formation decreased with increasing exposure time.

a single exposure period, thus simplifying the experimental measurements. Ideally, the initial rate at time zero is used, but an atypical condition exists during the initial time period (approximately 1 hour) while the various diffusion gradients are established. However, the dissolution or decalcification rate was close to being linear during the first 4 to 6 days, so that the relative effect of the different variables could be determined using a long time interval. A period of 0 to 96 hours was chosen in order to give the necessary latitude experimentally and yet approach the conditions of a true rate measurement as a function of initial conditions. This requires that neither the initial reaction or solution conditions have been altered enough nor the reaction products accumulated sufficiently to alter significantly the reaction rate.

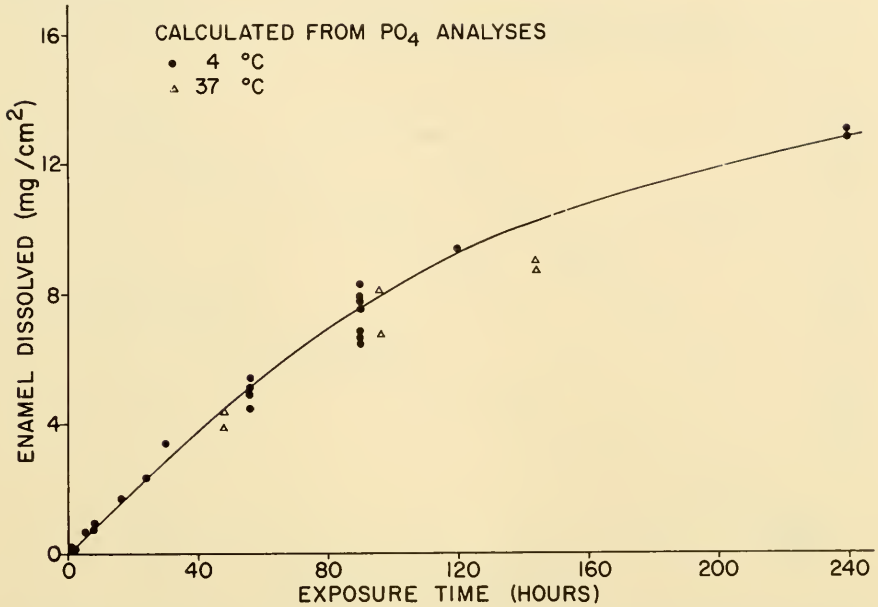


FIG. 26. Enamel dissolution as a function of time during exposure at 4°C and 37°C to a medium consisting of 0.05 M lactic acid and 0.03 M $CaCl_2$ plus 6 per cent hydroxyethyl cellulose (with low buffer content) adjusted to pH 3.5. Temperature had little effect on the enamel dissolution rate. The rate was decreased by addition of $CaCl_2$ (compare with Fig. 25).

The next series of observations deal with enamel dissolution rate (expressed as mg of enamel dissolved per cm^2 of enamel surface exposed per 96 hours exposure) as a function of hydrogen ion concentration (i.e., pH), lactate concentration, and calcium concentration (Figs. 28 to 33). These quantitative results are higher than the preceding measurements because a different sample of hydroxyethyl cellulose was used that contained a greater quantity of the buffer salt impurity. The results become comparable when the decalcification rate is plotted as a function of total acid.

The rate of enamel dissolution during incipient carious lesion formation increased with increasing hydrogen ion concentration. The rate is a nonlinear function of the hydrogen ion concentration, and the slope of the function decreases as the hydrogen ion concen-

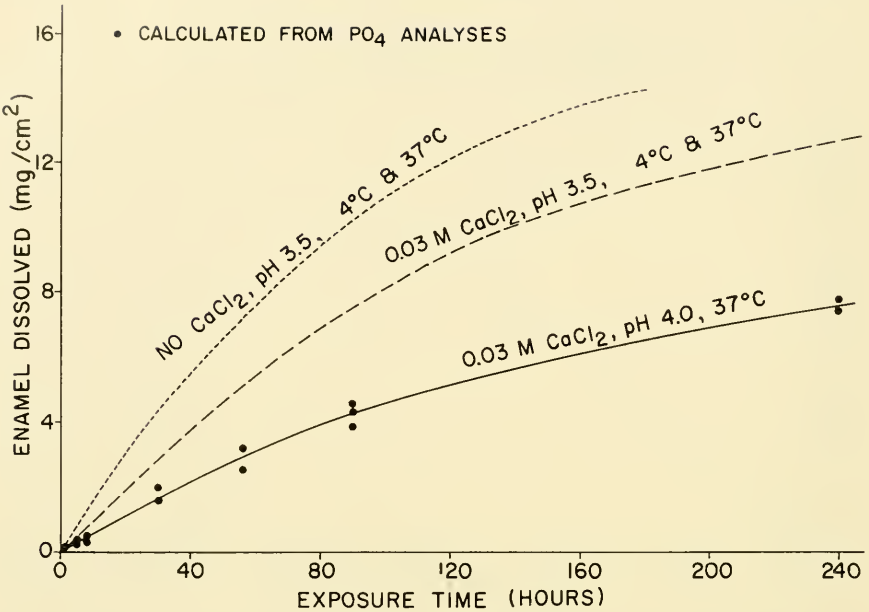


FIG. 27. Comparison of enamel dissolution rate as a function of time during exposure to mediums containing 0.05 M lactic acid plus 6 per cent hydroxyethyl cellulose (with low buffer content) at different $CaCl_2$ concentrations and pH values. Increasing the $CaCl_2$ concentration or the pH value decreased the rate of enamel dissolution during incipient carious lesion formation. The line of short dashes is from Fig. 25 and the line of long dashes is from Fig. 26.

tration is increased. The enamel dissolution rate was measured as a function of pH for a medium at 0.05 M lactate concentration and two different calcium chloride concentrations. The data are plotted as a function of hydrogen ion concentration (Fig. 28). Increasing calcium chloride concentration caused a decrease in the rate and hence in the slope of the rate curve.

The rate of enamel dissolution increased linearly with increasing lactate concentration at any pH level or calcium chloride concentration. The effect of lactate concentration on rate was determined for two different pH values and three different calcium chloride concentrations (Fig. 29). The slope of these linear plots varied with the different hydrogen ion and calcium chloride concentrations.

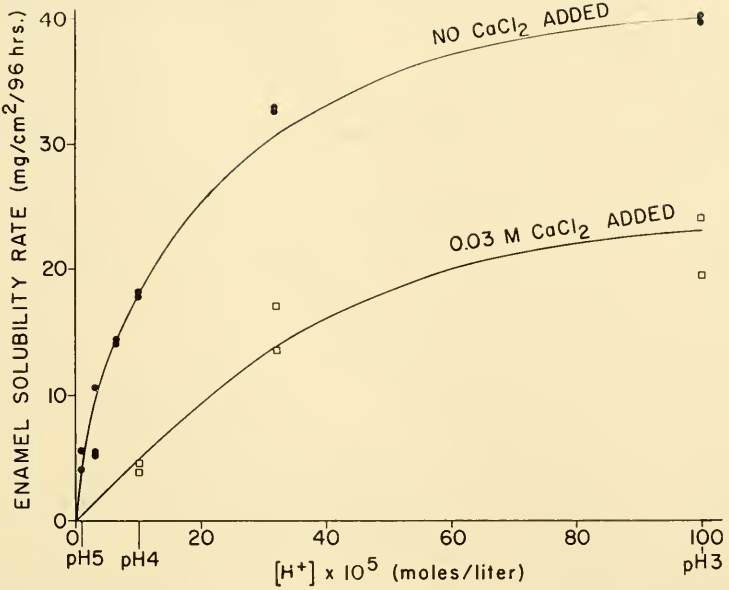


FIG. 28. Enamel dissolution rate as a function of hydrogen ion concentration during exposure at 37°C to a medium containing 0.05 M lactic acid plus 6 per cent hydroxyethyl cellulose with and without 0.03 M $CaCl_2$. In either case, the enamel solubility rate increased with increasing hydrogen ion concentration, but with a continuously decreasing slope. This latter effect is related to the amount of undissociated acid formed from the buffers. $CaCl_2$ caused a decrease in the rate of enamel dissolution.

Also, the rate did not fall to zero at zero lactate concentration, reflecting the buffer capacity of the hydroxyethyl cellulose sample.

The rate of enamel dissolution decreased with increasing calcium concentration, but not linearly (Fig. 30). However, replotting the data in logarithmic form resulted in a straight-line relationship (Fig. 31). The effect of calcium chloride on the dissolution rate was measured at two different pH levels and lactate concentrations. A logarithmic plot is a typical procedure for determining the order of reaction with respect to the variable under study. In this case, because of mathematical requirements, the logarithm of the difference in rate with and without calcium present is plotted versus the logarithm of the calcium chloride concentration. The slope of this plot gives the

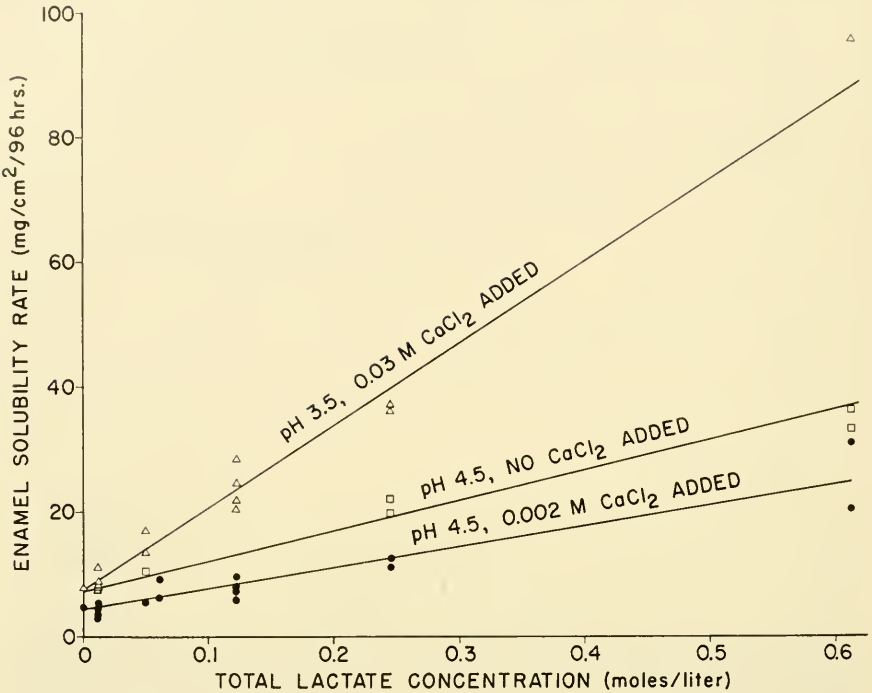


FIG. 29. Enamel dissolution rate as a function of lactate concentration during exposure at 37°C to a medium containing lactic acid and $CaCl_2$ plus 6 per cent hydroxyethyl cellulose adjusted to either pH 3.5 or 4.5. The rate was linear and increased with increasing lactate concentration. Decreasing pH or $CaCl_2$ concentration increased the slope of these linear functions. The intercept at zero lactate concentration resulted from the effect of the buffer content of the hydroxyethyl cellulose sample.

order, which appears to be equal to about ¼. The fractional aspect of the order is quite certain, but further data are required to establish the quantitative exactness.

It should also be pointed out that under conditions giving very high enamel dissolution rates, involvement of the surface became more and more extensive, leading to the condition, described by Hals *et al.* (1955), of an "inner" and an "outer" spot. However, there appeared to be no detectable discontinuity in the rate when this effect was encountered and included in the measurement of enamel dissolution (Fig. 29). The very highest rates resulted in severe sur-

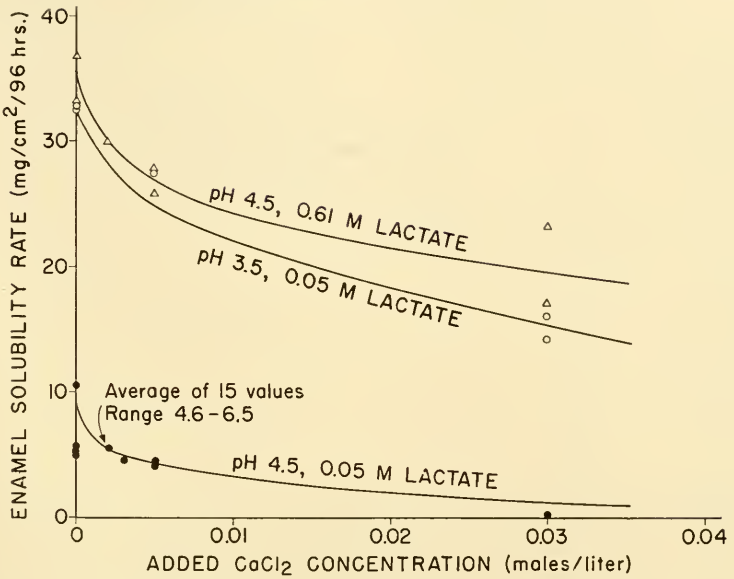


FIG. 30. Enamel dissolution rate as a function of $CaCl_2$ concentration during exposure at 37°C to a medium containing lactic acid and $CaCl_2$ plus 6 per cent hydroxyethyl cellulose. Addition of $CaCl_2$ decreased the rate at two different pH levels and lactate concentrations.

face dissolution (e.g., at pH 3.5, 0.61 M lactate, 0.03 M $CaCl_2$). The maximum rate of enamel dissolution before surface damage became detectable by visual or microscopic observation was about 20 mg of enamel per cm^2 of surface per 96 hours exposure. Although not all the levels of composition variables investigated gave ideal incipient carious lesions, some measurements outside of the conditions for producing a typical lesion were necessary in order to assess the effects within the preferred region.

The importance of undissociated acid in addition to the hydrogen ion for controlling the rate of enamel dissolution during incipient carious lesion formation has been suggested (Gray *et al.*, 1962). The recent results mentioned earlier (Kapur *et al.*, 1962) support this hypothesis. The data from the acid–organic polymer systems studied in this presentation were plotted versus total acid concentration (Figs. 32 and 33) to test this concept further. The undissociated lactic acid was calculated from the dissociation constant (Gray,

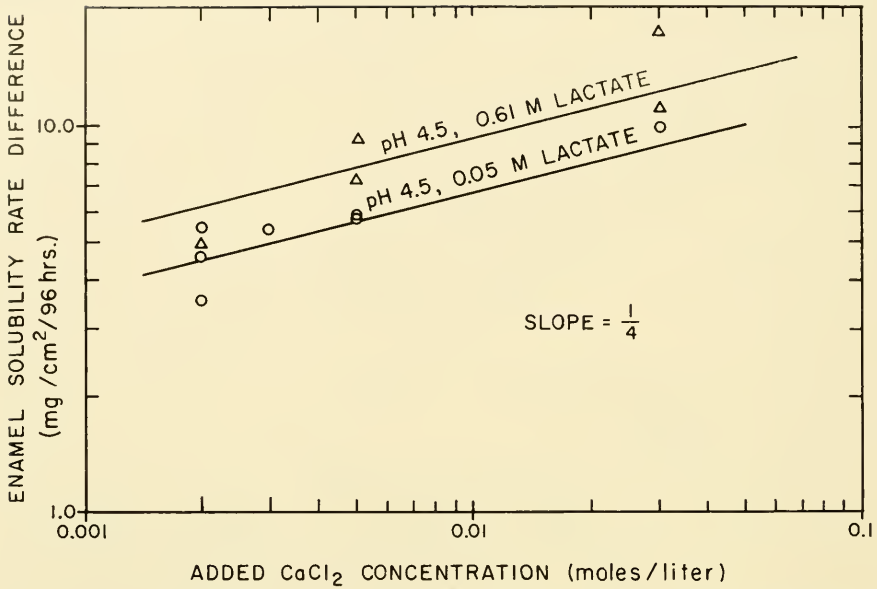


FIG. 31. A logarithmic plot of the data from Fig. 30 for the enamel dissolution rate during incipient carious lesion formation as a function of $CaCl_2$ concentration was a straight line. The rate was, therefore, proportional to the $CaCl_2$ concentration taken to a power equal to the slope of this plot.

1962) and the known lactate concentration which had been added. The undissociated acid content arising from hydroxyethyl cellulose was determined from pH titration curves. The hydrogen ion concentration was calculated from the pH measurement. The contribution of the various acid sources is given in Table II. It is immediately apparent that the rate of enamel dissolution over all pH values and lactate concentrations can be summarized by a single straight-line function of total available acid concentration for a given calcium chloride concentration. A separate linear function with a different slope is required for each calcium level.

In the absence of organic material, lactate buffer of any appreciable concentration destroyed the surface and only a slight advancing front of decalcification occurred (extending only 5 to 10 microns into the enamel for 0.05 M lactic acid at its "as is" pH of 2.6). The loss of enamel and, to some extent, the advancing decalcification front are apparent in a photomicrograph (Fig. 34) or in a micro-

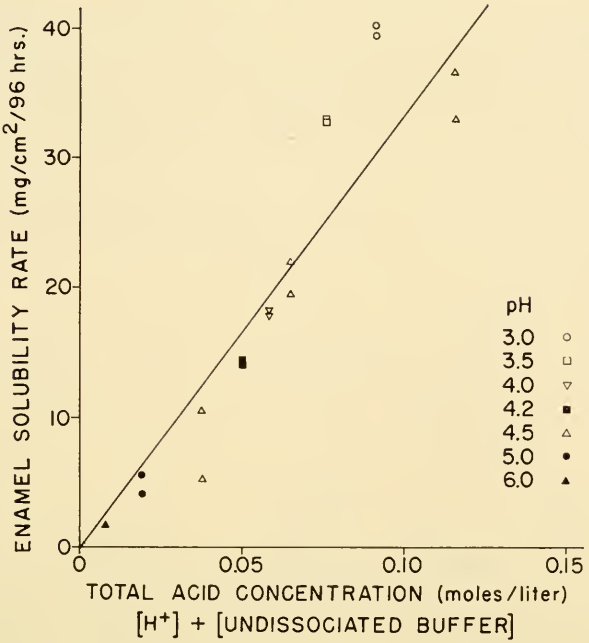


FIG. 32. The enamel dissolution rate during incipient carious lesion formation was a straight-line function of total acid concentration for a medium containing lactic acid plus 6 per cent hydroxyethyl cellulose over a pH range of 3 to 6 at 37°C. A very good lesion was obtained even at pH 6. Therefore, the rate can be expressed by a simple equation in terms of acid concentration (see equation 2, page 252).

radiograph (Fig. 35); the advancing front in this same sample can be seen in an electron micrograph (Fig. 36). The appearance of this defect in the electron micrograph was quite different from that of an incipient carious lesion (Fig. 22). As the concentration of total lactate was decreased at a constant pH value of 4.5 until only hydrogen ions were present (i.e., as HCl), the ratio of subsurface to surface dissolution increased (microradiograph, Fig. 37; photomicrograph, Fig. 3i), but surface dissolution was always present. The addition of calcium and phosphate ions to the acid solutions, still in the absence of organic polymer, further increased the ratio of subsurface to surface dissolution and permitted a higher acidic buffer concentration to be used (microradiograph, Fig. 38). The lesions

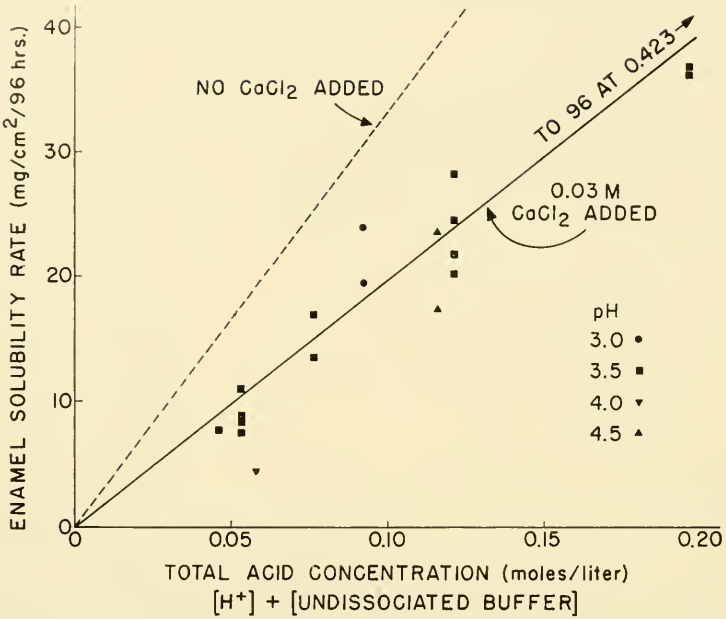


FIG. 33. The enamel dissolution rate was a straight-line function of total acid concentration for a medium at 37°C containing lactic acid plus 6 per cent hydroxyethyl cellulose with or without 0.03 M $CaCl_2$ added. The $CaCl_2$ inhibited the rate proportionally across all acid concentrations and pH values. The dashed line is taken from Fig. 32.

appeared to be excellent examples of incipient carious lesions, but again surface attack was never completely arrested. However, the character of the subsurface dissolution was completely different from that in the lesions produced *in vivo* or *in vitro* with the organic polymer–acid systems (Fig. 3j). The lesion was continuous to the surface with no relatively sound outer layer, and had no birefringence, conditions indicative of very advanced decalcification as defined by Gustafson (1957). Nevertheless, further studies of such systems should provide additional valuable information concerning the dynamics and equilibria of incipient carious lesion formation once correlation with the organic polymer–acid system has been established.

The decalcification rate of enamel during incipient carious lesion

formation also depended on the size and shape of the enamel surface area that was exposed to the decalcification medium. Experimentally, this means that for direct quantitative comparisons, all samples should be of the same size; 15 mm^2 samples were used throughout the present study. The rate per unit area increased linearly with

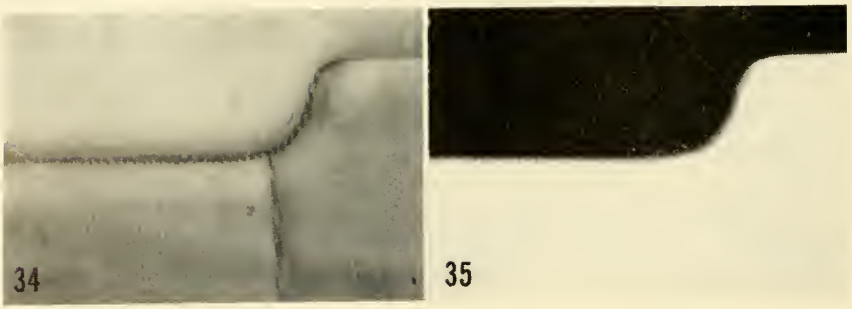
TABLE II. Acid Concentration of Decalcification Medium in Moles per Liter

Total lactate	pH	$[H^+]$	Undissociated lactic acid[a]	Undissociated acid in hydroxyethyl cellulose[b]	Total acid[a] (dissociated + undissociated)
0.05	3.0	10×10^{-4}	0.042	0.050	0.093
0.00	3.5	3.2×10^{-4}	—	0.045	0.045
0.01	"	"	0.006	"	0.051
0.05	"	"	0.031	"	0.076
0.12	"	"	0.074	"	0.119
0.25	"	"	0.154	"	0.199
0.61	"	"	0.376	"	0.421
0.05	4.0	1.0×10^{-4}	0.017	0.042	0.059
0.05	4.2	0.64×10^{-4}	0.012	0.039	0.051
0.00	4.5	0.32×10^{-4}	—	0.031	0.031
0.01	"	"	0.001	"	0.032
0.05	"	"	0.007	"	0.038
0.06	"	"	0.008	"	0.039
0.12	"	"	0.017	"	0.048
0.25	"	"	0.034	"	0.065
0.61	"	"	0.084	"	0.115
0.05	5.0	0.1×10^{-4}	0.002	0.017	0.019
0.05	6.0	0.01×10^{-4}	0.000	0.005	0.005
0.61	"	"	0.003	"	0.008

[a] Calculated and checked by titration.

[b] Determined by titration. The other sample of low buffer content contained undissociated acid of 0.026 M at pH 3.5 and 0.022 M at pH 4.5.

decreasing sample area, approximately doubling for a 50 per cent reduction in surface area (Fig. 39). The effect could be the result of the greater volume available for diffusion of reactant and reaction products to and from the enamel at the sample edges as compared with the middle of the sample, and, therefore, would be a function of the perimeter of the sample.



FIGS. 34 AND 35. A section of enamel after exposure for 7 hours to an unagitated aqueous solution of 0.05 M lactic acid at its natural pH of 2.6. The right side was protected with varnish to serve as a control.

Fig. 34. A light micrograph shows the complete loss of enamel to a depth of 50 microns and the damage extending slightly into the remaining sample. (× 2000.)

Fig. 35. A microradiograph also shows the complete loss of the enamel surface and confirms the presence of a decalcification front. (× 2000.)

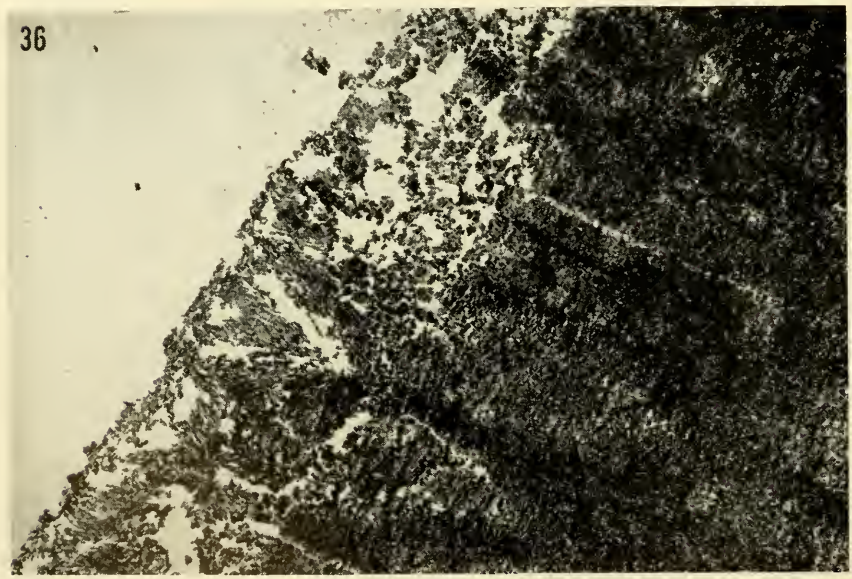


FIG. 36. An electron micrograph of a section cut from the sample of Figs. 34 and 35. This enamel sample was exposed 7 hours to an aqueous solution of 0.04 M lactic acid at the "as is" pH of 2.6. Although most of the enamel was dissolved away as shown in Figs. 34 and 35, a slight advancing front of decalcification extended into the enamel about 5 microns. (× 5000.)

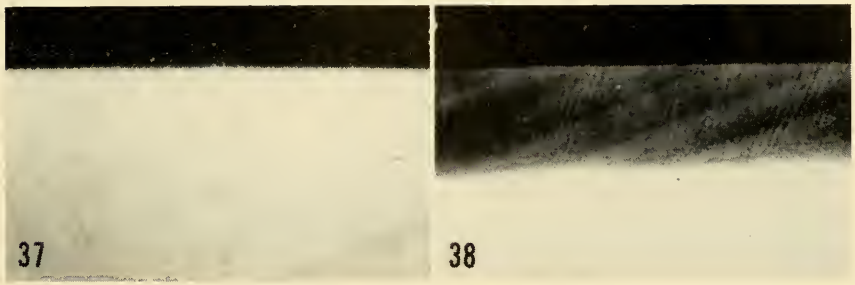


FIG. 37. A microradiograph of a section of enamel after a 15-day exposure at room temperature to an aqueous solution of hydrochloric acid at pH 4.5 shows that decalcification had occurred near the surface but had not penetrated very far. Examination of the surface with a metallurgical microscope showed that light pitting had occurred. (× 200.)

FIG. 38. A microradiograph of a section of enamel after a 15-day exposure at room temperature to an aqueous solution of 0.0005 M $CaCl_2$ and 0.0005 M NaH_2PO_4, adjusted to pH 4.5, shows that extensive decalcification had occurred. The subsurface dissolution penetrated quite far but was fairly uniform throughout the damaged region. The surface was much pitted by the exposure, but this could be seen only with a metallurgical microscope. (× 200.)

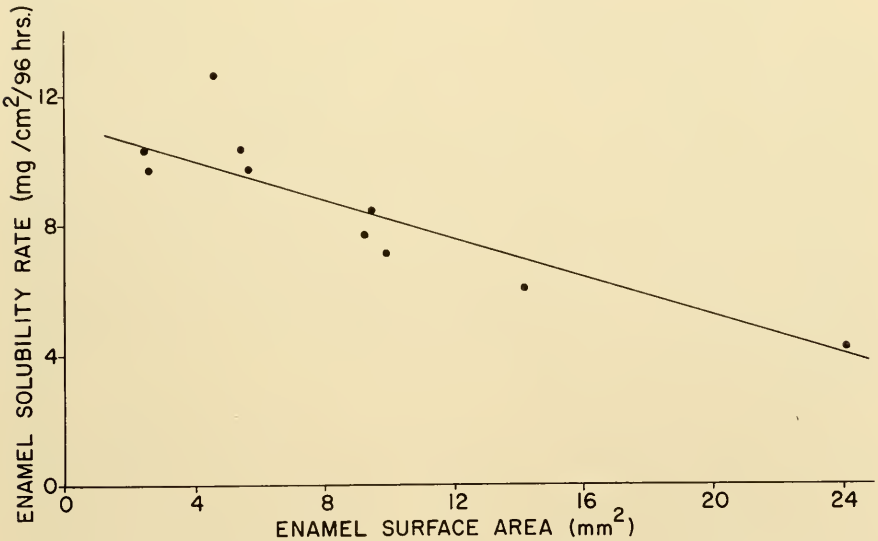


FIG. 39. The enamel dissolution rate was measured in a medium at 37°C consisting of 0.05 M lactic acid and 0.002 M $CaCl_2$ plus 6 per cent hydroxyethyl cellulose adjusted to pH 4.5, using enamel samples with different surface areas. The smaller the surface area, the faster was the absolute rate of dissolution. This effect is a result of the geometry of the experimental set-up and is probably related to diffusion conditions.

Important Factors in Incipient Carious Lesion Formation

Preservation of Enamel Surface

In a previous section, it was indicated that the enamel surface is protected principally by the presence of polymeric organic material. The exact nature of this protective mechanism is not understood, but a protective coating resulting from adsorption of the organic compound on the apatite crystals appears most logical. The higher organic content of incipient carious lesions as compared with normal enamel demonstrates that organic substances diffuse into these enamel defects. Therefore, protective coatings of organic origin can be expected within the enamel near the surface, and thus could account for the relatively sound outer layer overlying the main decalcified region. Indeed, electron micrographs do show that this outer layer has the highest residue after demineralization.

Obviously, some dissolution occurring in this relatively sound outer layer would promote the diffusion of acid and organic material within the enamel. The reduced density of the outer layer of a lesion produced *in vitro* can be seen in microradiographs.

In previous studies (Soni and Brudevold, 1959), it has been shown quite clearly that this outer layer in natural lesions has a lower density than normal enamel. The hardness is also slightly reduced within this layer (Gustafson, 1957), consistently with the reduced density. In electron micrographs, the channels between the rods going through this layer are clearly apparent. The organic material apparently diffuses into the enamel as the acid creates voids and enlarges the interrod spaces.

In order for the organic material to be protective (Table I), it must be water soluble or in a colloidal suspension so as to gain access to the hydroxyapatite surface, and it must be adsorbed. Polymeric saccharides and proteins are known to adsorb on many substrates, and these materials are highly effective in protecting the enamel surface during subsurface decalcification. The monomers have little or no such effect. The organic material, constantly present and dif-

fusing into the enamel through the enlarged pathways, will begin, at some point, to have a larger and larger effect in reducing the attack within the enamel lesion as more and more exposed apatite crystals become covered with the polymeric substances. For this reason, it is also very doubtful whether the organic component can ever be completely omitted in an *in vitro* system for reproducing the typical natural lesion.

The protection of the surface layer is increased by the presence of acid-insoluble inorganic compounds, particularly those resulting from reaction with enamel, such as calcium fluoride or stannous phosphate.

Diffusion within the Enamel

In a system which restricts interaction with the enamel surface, hydrogen ions will tend to diffuse into any region lower in acid concentration. That such regions must exist and are accessible to a solution phase has been demonstrated by the diffusion of radioactive isotopes through enamel under *in vivo* conditions (Sognnaes *et al.*, 1955). It is obvious from experimental results that although the surface apatite crystals are protected by the organic polymers, the diffusion of acid into enamel is not blocked by this coating. Hence, any acid penetrating beyond the limits of the organic protective coating will react to dissolve subsurface enamel. At the point of reaction between acid and hydroxyapatite, the principles involved in the surface dissolution of enamel in acid, as determined in a previous study (Gray, 1962), can be invoked. As the acid reacts at specific sites in the enamel, calcium and phosphate accumulate and retard or arrest the dissolution reaction. Then, acid will tend to diffuse farther into enamel for reaction at advanced unprotected sites, but it is limited by the available diffusion pathways and the depleted acid concentration. Diffusion of soluble calcium and phosphate out of enamel is favored by the wide diffusion pathways already established and by the favorable concentration gradient to the exterior solution. At the same time, any increase in pH, either ahead of or behind the reaction site, will cause reprecipitation of calcium phosphate, the particular phase depending on the existing conditions. As calcium

and phosphate concentration is reduced by precipitation and/or diffusion, the reaction with hydrogen ion is renewed and the lesion below the surface slowly progresses.

Concentration Gradients in the Solution Phase

In the course of incipient carious lesion formation, different solution compositions must exist inside and outside the enamel with a gradient extending from one to the other. For the *in vitro* system the external or bulk solution is defined by the initially prepared composition. At or near the enamel surface, however, there will be a region of different composition as solution components diffuse into the enamel and products diffuse out. The hydrogen ion and undissociated acid concentrations will certainly be lower and the calcium and phosphate concentrations (from the reaction products) will be higher at the surface than in the external bulk solution. Inside the enamel, the hydrogen ion concentration will be lower than in the bulk solution, owing to reaction of hydrogen ions with enamel. Defining the concentrations of reaction products relative to the exterior bulk solution is not always so straightforward. When such materials are not incorporated initially in the decalcification medium, the concentration within the enamel will obviously be higher. When, however, calcium and phosphate are added initially as part of the decalcification medium, sometimes in large amounts, the concentrations in the exterior phase may approach or even exceed those in the solution phase inside the enamel. As the concentration of calcium, for instance, is raised in the external decalcification medium, the rate of enamel dissolution decreases owing to the decrease in rate of removal of the reaction products; it will be recalled that diffusion rate is controlled principally by the difference in concentration from one site to the other. At sufficiently high calcium concentrations, the enamel dissolution reaction stops altogether.

The amount of calcium required in the decalcification medium to inhibit incipient carious lesion formation is greater than would be expected on the basis of enamel solubility rate measurements (Gray, 1962). The existence within the enamel of other species of calcium and phosphate in the form of soluble complexes, for example acid phosphate and calcium lactate, would permit a greater rate of out-

ward diffusion, in spite of the high concentrations of calcium and phosphate in the exterior solution. The dissociation, or association, of these complexes under the different conditions existing in the external solution would allow this process to continue.

It should also be pointed out that the conditions of the solution phase will determine the solid phase, which will either reprecipitate and/or exist at any equilibrium that is attained.

Chemical Kinetics and Equilibrium

Kinetics of Dissolution

In the presence of an acid maintained at a constant concentration, the rate of enamel dissolution, as well as the various concentration gradients just described, will shift only gradually but continuously as the mineral substrate breaks down. It is this pseudo steady state that can be measured quantitatively and for which data have been presented. If the acid supply increases and decreases, as would be expected under oral conditions, there will occur a cycling of events that is difficult to describe mathematically. Consequently, only the condition of a single, continuous exposure will now be considered and treated mathematically.

It has been shown that the rate of incipient carious lesion formation is determined largely by the rate of reaction of acid with the inorganic portion of enamel. The small effect of temperature on rate of enamel dissolution during incipient carious lesion formation supports the concept of diffusion control. As shown above, the rate depends on total available acid concentration, but hydrogen ion contributes little directly to the rate because of its low concentration. The undissociated acids, always relatively high in concentration as compared with hydrogen ions, provide the hydrogen ions for reaction through diffusion to and dissociation at the apatite crystal surface. The linear relationship of the enamel dissolution rate as a function of total acid concentration shows that the rate of dissolution is "first order" with respect to acid concentration (Figs. 32 and 33).

The dissociated buffer anion appears to have no direct controlling effect on the rate of dissolution during incipient carious lesion formation. The linear relation of the plot of Fig. 29 supports this conclu-

sion, as deviations from linearity would be expected if either inhibition or enhancement were involved. The anion could have been expected to change the rate because of its effect on dissociation of lactic acid or because of its weak calcium complex (Bjerrum, 1957).

Mathematically, the rate of incipient carious lesion formation (i.e., the enamel dissolution during incipient carious lesion formation) can be expressed as follows, using the usual first order rate expression (Laidler, 1950), assuming that the buffer anion is inert and all other conditions (e.g., calcium and phosphate concentrations) are constant:

$$\frac{dEn}{dt} = k_0[H^+] + k_1[HB] \tag{1}$$

where

dEn/dt = rate of enamel dissolution during incipient carious lesion formation (mg enamel/cm^2/96 hrs.)
$[H^+]$ = hydrogen ion concentration (moles/liter)
$[HB]$ = undissociated acid concentration (moles/liter)
k_0, k_1 = constants

For combinations of several different buffers, the expression would take the following general summation form:

$$\frac{dEn}{dt} = k_0[H^+] + \sum^{m} k_m[HB]_m \tag{2}$$

Then, for any buffer system and in terms of total buffer concentration (for ease of calculation), the following expressions (3 and 4) for the total buffer concentration $[TB]$ and the dissociation constant K of the buffer are substituted in equation 2:

$$[TB] = [HB] + [B^-] \tag{3}$$

$$K = \frac{[H^+][B^-]}{[HB]} \tag{4}$$

where

$[B^-]$ = dissociated acid buffer anion concentration (moles/liter)

The result of the substitution is the following equation:

$$\frac{dEn}{dt} = k_0[H^+] + \sum^{m} k_m \frac{[H^+][TB]_m}{K_m + [H^+]} \tag{5}$$

If the strength of the buffer decreases (i.e., the dissociation constant decreases), the rate should increase, reach a maximum, and then decrease. For most practical cases, the first term as well as the $[H^+]$ in the denominator of the second term can be ignored since they are small, and then:

$$\frac{dEn}{dt} = k_1 \frac{[H^+]}{K} [TB] \tag{6}$$

For these expressions, all other variables are assumed to be constant, such as the cation and organic polymer concentration of the decalcification medium. To incorporate these other variables into the function requires additional terms which must be added to or multiplied by the existing terms. The preceding equation, however, is the fundamental expression of the rate of incipient carious lesion formation.

Calcium suppresses the rate, and this inhibition can be expressed by including a term, $k[Ca]^n$, in the immediately preceding equation. The value of the exponent n is determined from the slope of the logarithmic plot (Fig. 31, $n = \frac{1}{4}$) of the rate versus calcium concentration. It has not yet been determined how best to incorporate this additional term to augment the fundamental expression for rate of formation of incipient carious lesion.

Phase Identification by Equilibrium Solubility Studies

The mechanism of rate inhibition by calcium is complicated but is almost certainly not a reversal of the reaction to re-form hydroxyapatite (i.e., common ion effect) (Gray, 1962). One reason for this conclusion is that phase diagram data (Van Wazer, 1958) indicate that dicalcium phosphate, not hydroxyapatite, will be the existing phase under the conditions present during enamel dissolution. Another supporting fact is that many, if not all, cations forming insoluble phosphate salts have a similar repressing effect (although to widely different degrees), leading to the conclusion that inhibition is a generalized effect of cations. To a much more limited extent, similar effects can be expected and are found for anions, particularly those which form insoluble calcium salts.

At present, the only mechanism by which all the different effects observed experimentally can be satisfactorily explained (Gray *et al.*, 1962) is the precipitation of some of the reaction products as phases that are different from the original hydroxyapatite. These phases form on the hydroxyapatite surface and limit further reaction or, at equilibrium, completely prevent access of hydrogen ions to the enamel or egress of calcium and phosphate ions to the bulk solution phase. The existence of these phases has been proved by equilibrium solubility measurements, as will now be described.

Detection of the surface phases as described in the previous paragraph by currently available physical methods is very difficult because, in the first place, the layers formed are only a few angstrom units thick (approximately 25 A), on the basis of calculations from chemical and physical data. Secondly, isolation of the solid samples is at present impracticable because any change of the equilibrium solution in contact with the surface will tend to change the phase. If, however, the original postulation is correct, then the solution in equilibrium with the surface coating should have a composition satisfying the solubility product for the phase existing on the hydroxyapatite surface. These measurements of solubility product were made in simple acidic solutions (i.e., not decalcification mediums). Briefly, the experimental work was as follows (Francis, in preparation). Samples of dental enamel (human and bovine, both intact and powdered) and synthetic hydroxyapatite (Victor Chemical Works) as well as dicalcium phosphate were agitated in solutions of lactic or acetic acid buffers in a pH range from 4.6 to 6.5. The slurries were filtered or centrifuged to remove the solids, and the solution pH, calcium concentration, and phosphate concentration were measured. Determinations as a function of exposure time were made to confirm that equilibrium conditions had been attained. Corrections for complexing of calcium by lactate and acetate anion species were required, but ionic strength corrections could be ignored. Several ratios of solution to solid, and addition of calcium (as $CaCl_2$) and phosphate (as NaH_2PO_4) initially to the acid solution, were also studied. Regardless of conditions, the K_{sp} consistently observed was that of dicalcium phosphate (Van Wazer, 1958), as summarized in Table III. This proved that the solution was in equi-

librium with a phase different from hydroxyapatite, which must, therefore, be covered by this other phase.

Previous work (Gray *et al.*, 1962) has shown that the initial solubility rates of normal enamel and fluoridated enamel were the same.

TABLE III. Equilibrium Solubility Constants Determined from Synthetic and Natural Phosphates in Acid Buffer Solutions

Solid	Buffer concentration	Solid solution ratio	pH range	K_{SP}[a] ($[Ca^{++}][HPO_4^{=}]$)	
Dicalcium phosphate	0.20 M acetate	0.1	4.7–6.7	5.4×10^{-7}	5.3×10^{-7}
Dicalcium phosphate	0.18 M acetate	0.1	5.1	4.7×10^{-7}	
Dicalcium phosphate	1.0 M acetate	0.1	4.8–4.9	5.5×10^{-7}	
Synthetic apatite	0.20 M acetate	0.1	5.0–5.3	2.7×10^{-7}	4.5×10^{-7}
Synthetic apatite	0.20 M lactate	0.1	4.6	8.1×10^{-7}	
Synthetic apatite	1.0 M acetate	0.1	4.8–4.9	4.5×10^{-7}	
Synthetic apatite	0.20 M acetate	0.01	4.9–5.2	3.8×10^{-7}	
Synthetic apatite	1.0 M acetate	0.01	4.9	4.4×10^{-7}	
Synthetic apatite + $CaCl_2$	0.18 M acetate	0.001	4.8, 5.1	1.5×10^{-7}	
Synthetic apatite + NaH_2PO_4	0.18 M acetate	0.001	5.0, 5.1	6.5×10^{-7}	
Bovine incisor enamel	0.20 M acetate	0.1	5.0–6.2	5.0×10^{-7}	5.5×10^{-7}
Bovine incisor enamel + $CaCl_2$ + NaH_2PO_4	0.20 M acetate	0.1	4.9	4.5×10^{-7}	
Bovine incisor enamel	0.20 M acetate	0.01	4.9	7.8×10^{-7}	
Bovine incisor enamel	0.20 M lactate	0.01	5.9	4.2×10^{-7}	
Bovine incisor enamel + $CaCl_2$	0.20 M acetate	0.01	5.0, 5.2	6.1×10^{-7}	
Bovine incisor enamel + $CaCl_2$ + NaH_2PO_4	0.20 M acetate	0.01	4.9, 5.0	5.1×10^{-7}	
Bovine incisor enamel + NaH_2PO_4	0.18 M acetate	0.001	5.0	6.1×10^{-7}	
Intact human incisor enamel	0.20 M acetate	—	4.4	8.3×10^{-7}	7.1×10^{-7}
Human molar enamel and dentin	1.0 M acetate	0.1	5.1	12.0×10^{-7}	
Human molar enamel and dentin	1.0 M acetate	0.01	4.8	8.0×10^{-7}	
Human molar enamel and dentin	1.0 M acetate	0.004	4.9	3.7×10^{-7}	
Human molar enamel and dentin	0.18 M acetate	0.002	5.1	3.0×10^{-7}	
Rat bone	1.0 M acetate	0.01	5.0	12.0×10^{-7}	8.5×10^{-7}
Rat bone	0.18 M acetate	0.002	5.2	4.8×10^{-7}	
Human enamel	Saliva + lactic acid (Fosdick and Starke, 1939)			4.4×10^{-7}	4.1×10^{-7}
Synthetic apatite	HCl + NaCl (Ericsson, 1949)			2.8×10^{-7}	
Synthetic apatite	Saliva + lactic acid (Ericsson, 1949)			4.5×10^{-7}	

[a] The value of the K_{SP} determined in the present work across all systems (85) was $5.4 (\pm 2.7) \times 10^{-7}$. An average value of $4.1 (\pm 2.0) \times 10^{-7}$ was obtained from data which included 61 systems from the literature.

Fluoridated enamel became less soluble only after fluoride accumulated in the acid solution, suggesting that inhibition resulted from the deposition of calcium fluoride from the reaction products onto the enamel surface. Furthermore, there is no fundamental reason

why fluorapatite should have a lower initial rate of solubility in acid than hydroxyapatite when the reaction of hydrogen ion with the phosphate group must be responsible for the breakdown of the apatite lattice. Measurements of the solution phase in equilibrium with fluorapatite mineral in acidic buffers gave the solubility product for calcium fluoride (Table IV). The measured solubility product of

TABLE IV. Equilibrium Solubility Constants of Calcium Fluoride and Fluorapatite Determined in Lactate Buffer

Solid	Buffer concentration	Solid/solution ratio	pH	K_{SP}[a] ($[Ca^{++}][F^{-}]^2$)
CaF_2	0.20 M	0.1	3.4, 4.3	2.7×10^{-10}
CaF_2	0.47 M	0.01	3.5	3.9×10^{-10}
Synthetic fluorapatite	0.20 M	0.1	4.0	4.7×10^{-10}
Synthetic fluorapatite	0.20 M	0.01	4.6	2.5×10^{-10}
Asparagus stone[b]	0.20 M	0.002	3.5	2.0×10^{-10}
Intact asparagus stone[b]	0.20 M	—	3.5	3.4×10^{-10}

[a] The value of the K_{SP} determined across all systems (10) was 2.8 (± 1.2) $\times 10^{-10}$.
[b] A naturally occurring mineral form of fluorapatite [$Ca_{10}(PO_4)_6F_2$].

calcium fluoride in lactate buffer is given for comparison because it was found that the value was lower, corresponding to literature values, when determined in water or unbuffered acidic solutions.

Although pH of the solubilizing solution and composition of the enamel may have only an indirect effect in determining how fast enamel dissolves (or an incipient carious lesion forms), they will have a major role in determining the phase at any equilibrium and, therefore, the rate at which the equilibrium is reached. Furthermore, it is necessary to determine the fate of the phosphate phase laid down during a low pH condition when the external pH conditions return to values once more establishing apatite as the stable phase. Unfortunately, solubility product measurements cannot answer this question, but new physical techniques, such as back-scattering electron diffraction and infrared absorption spectroscopy by attenuated total reflection, give promise of confirming the presence of these thin coatings which have been discovered by chemical means.

Among the problems which remain to be solved in the description

of the formation of incipient carious lesions are: the positive identification of the organic fractions in the lesion; the events leading to the relatively sound outer layer of enamel, especially with a view to separating the effects of by-passing and reprecipitation; diffusion measurements through enamel, particularly relating to transport of acid and reaction products; and the role of acidic forms of phosphate as well as the various weak complexes of calcium with phosphate, bicarbonate, and lactate in the transport of the reaction products.

Summary

Incipient carious lesions of enamel, typical of those formed *in vivo*, have been produced by purely chemical systems. These systems were used to investigate the variables affecting incipient carious lesion formation and elucidate the mechanisms involved.

The essential factors necessary for the formation of incipient carious lesions are (1) a reactant (acid) to dissolve the hydroxyapatite and (2) an organic polymer to protect the external surface of the enamel. The inclusion of the reaction products—calcium and phosphate—has mediating effects ranging from complete inhibition of dissolution to subtle alterations in subsurface decalcification, permitting closer approach to duplication of the natural lesions.

Incipient carious lesion formation can be explained for the general case by the dynamics of the heterogeneous system consisting of an acidic solution and a calcium phosphate solid. This involves chemical phase transformation, equilibria, and kinetics. The rate of enamel dissolution during incipient carious lesion formation is directly proportional to total acid concentration (dissociated plus undissociated), a "first order" diffusion-controlled reaction. This reaction can be inhibited or brought to equilibrium by the presence of ions which form protective deposits on apatite surfaces as salts of the reaction products, such as dicalcium phosphate or calcium fluoride.

The existence of dicalcium phosphate on the surface of enamel, bone, and synthetic hydroxyapatite, and the existence of calcium fluoride on the surface of fluorapatite, in equilibrium with acid solutions, has been established.

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9

Factors Influencing the Initiation, Transmission, and Inhibition of Dental Caries

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THE discussion which follows represents observations, speculations, questions, and a few pertinent criticisms. In an attempt to present a broader perspective on dental caries we have tried to scan facets of the problem and not to focus narrowly on any special one. Our ultimate objective is to gain greater insight, to open doors, to reexamine postulations, to challenge concepts, and not to forge any idea into a fact. We feel that the emphasis belongs on a better understanding of the biology of caries and not on arguments over hypotheses.

Dental caries is a complicated infection which, for the most part, can be considered to originate outside the body proper. Lesions begin as a demineralization of enamel, a tissue of ectodermal origin, and later penetrate into the dentin and pulp, both of mesodermal origin. For many years most investigators of the disease have recognized that the substrate upon which the pathogenic bacteria subsist is composed largely of ingested food substances which come into contact with the teeth when conditions are favorable. Much attention has been directed to the role of acidogenesis, lactobacilli, and

the mechanisms of carbohydrate degradation and associated phosphorylation. It seems reasonable to expect that the exploration of some of the non-acidogenic aspects of the disease might lead to information of value in strengthening a program of comprehensive therapeutic control.

Students of dental caries recognize that in addition to salivary components, the highly variable nature of such diverse substrates as food residues and mineralized tissue makes the disease more complex to study than many other infections in which the intra- and extracellular components of the tissues furnish the principal substrate for the invading organisms. Some of the experimental findings to be discussed lend support to the idea that colonization and invasion of the cariogenic flora may be related to properties of the enamel and dentin which the pathogens find beneficial to their life cycle.

Etiological Factors

There is no single cause of caries, it is a multifactorial disease as are other infections. Etiology can be considered in terms of contributing factors in the host, the oral microbiota, and the dietary substrate. It is possible to gain a broader perspective on the disease processes in caries by considering somewhat analogous interactions between host, microbe, and over-all environment in other experimental infections, e.g. tuberculosis (Ratcliffe *et al.*, 1953, 1957). Additional insight into the complexities of biological interactions and into their analysis can be gained from the discourse "Cause and Effect in Biology" (Mayr, 1961).

In Fig. 1, three overlapping circles have been used to depict the possible relationships in the factorial triad contributing to caries activity, i.e. the host, the microbiota, and the diet. When the disease is active, these three sets of factors are in concentricity. Investigators are becoming increasingly aware that each one of these components behaves in a dynamic and variable way and that under laboratory conditions only the most delicate balance of the three will induce the disease and favor its progression. However, the magnitude of each contributing factor is still difficult to identify positively and is rarely amenable to control by conventional experimental methods.

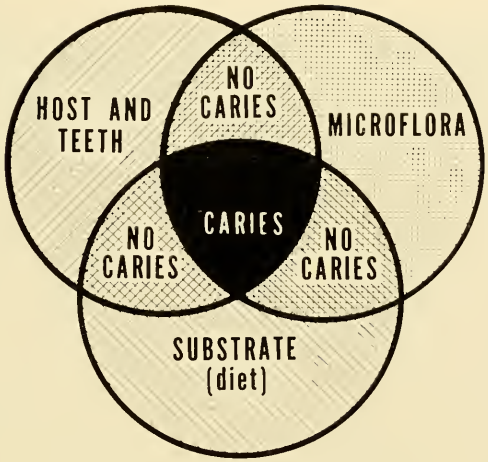

FIG. 1. Three overlapping circles depict the possible relationships of the factorial groups responsible for caries activity. There are numerous variables in each parameter. When the infection is active, conditions in each are conducive; but when it is inactive, to know which of the groups is in a noncontributory status is exceedingly difficult and often impossible.

Despite the prevalence of caries in human populations, we might also expect to find that rather specific conditions are necessary for activity. It follows, therefore, that in order to establish the caries-inactive host as immune or resistant, one must be sure about which factors are in a noncontributory status; such definition has rarely, if ever, been attained with certainty.

Many experiments in the field of dental caries have not been controlled in terms of the single-factor variable described in their design, and as a result such studies are open to various interpretations in terms of mechanisms responsible for the results. As suggested above, the limiting factor is obviously in the biological complexities of the disease itself rather than in the experimental design as it has been conceived in the mind of the investigator. "In view of the high number of multiple pathways possible for most biological processes, and in view of the randomness of many of the biological processes, particularly on the molecular level, causality in biological systems is at best only statistically predictive" (Mayr, 1961).

Host and Teeth

Although factors in the host and teeth are known to contribute importantly to caries activity, these are undoubtedly the most difficult to determine in a way that can be considered definitive. There are many factors in the oral cavity and in the teeth themselves which are thought to be either conducive or nonconducive to caries activity. Some of these are obviously related to the inherent nature of the host and to his over-all constitutional make-up. Of great importance and particular interest are those nonconducive factors which gradually develop within the host himself despite an inherent tendency toward susceptibility. For example, the older host, both animal and human, seems to be more refractory to this disease. In part this has been attributed to maturation factors in the teeth and to the presence of fluoride in the enamel. More information is becoming available as to what these properties may be in terms of chemical composition and physical properties (Brudevold, 1962*a*; Johansen, 1962; Zipkin *et al.*, 1962).

Dietary Substrate

The substrate formed by the ingesta and saliva provides a source of nutrient both to the macroorganism (host) and to the many microorganisms of the mouth and alimentary canal. If some of the bacteria (parasites) have a cariogenic potential, and if the food substrates are of the proper quality and quantity, colonization and plaque formation occur on certain coronal surfaces of the teeth. See Fig. 2. The microflora associated with caries does not induce lesions unless carbohydrates are present, but the cariogenic potential of carbohydrates in both animals and humans is related to such factors as quality, quantity, and physical consistency (Stephan, 1948; Haldi *et al.*, 1953; Gustafsson *et al.*, 1954; Shaw and Griffiths, 1960; Volker, 1962), and the carbohydrates must also be present in the mouth a certain length of time (Kite *et al.*, 1950; Larson *et al.*, 1962). These same conditions probably apply to other essential nutrients and not to carbohydrates alone.

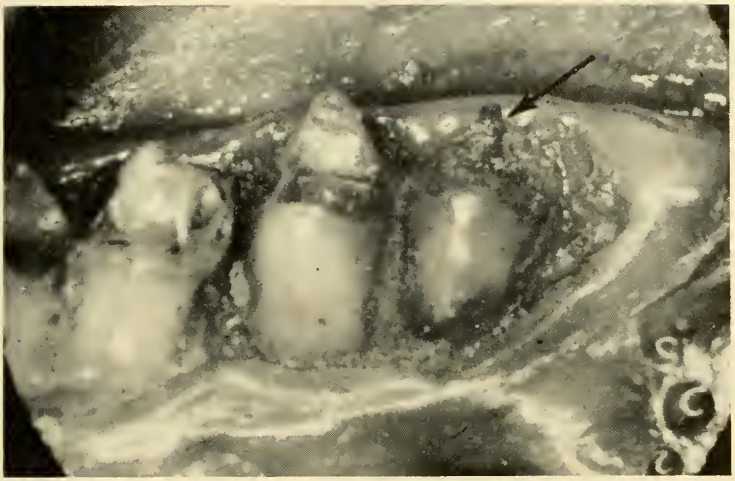

FIG. 2. When young hamsters harbor cariogenic microorganisms, colonization (plaque formation) rapidly develops on the coronal surfaces of the teeth. The photograph above was taken of a living animal to show the plaque which had covered the lingual sulcus and parts of the crown of the maxillary second molar. Bacterial activity in this accumulation had largely destroyed the distobuccal cusp of this tooth (arrow) about 3 weeks after commencement of an appropriate diet.

It is evident that there are various levels at which nutrients will be (1) adequate for the host and inadequate for the microflora, (2) adequate for the microflora and inadequate for the host, (3) unfavorable for both, or (4) *satisfactory for both.* See Fig. 3. The studies of Shaw (1949) and Shaw and Griffiths (1960) show that an experimental diet can be adequate for growth, reproduction, and general health of the host and at the same time favor bacterial activity associated with caries. Although some workers have suggested a different interpretation, the available evidence indicates little association between nutritional status and dental caries. This holds for animal studies and also for human populations (Russell *et al.*, 1961). In the interest of clarity and scientific accuracy, the question is whether one should accept the nutrition-caries interrelationship on exceedingly tenuous grounds or emphasize the importance of eating habits and dietary factors as they really are.

The property of food substrates which has been investigated for

INGESTA-SALIVA SUBSTRATE

COMPOSITION and/or QUANTITY (Chemical Physical)	(Proportion Duration)
HOST	PARASITE(S)
1. Adequate	Inadequate
2. Inadequate	Adequate
3. Inadequate	Inadequate
4. Adequate	Adequate

FIG. 3. Correlations between nutrition and caries activity are exceedingly difficult because it is necessary to recognize that the requirements of macroorganism (host) and microorganisms (parasites) may be either alike or different.

"cariogenicity" most frequently is acidogenic potential (Bibby, 1961; Mörch, 1961). Although the statement that "carbohydrate foodstuffs are the principal causative agents of dental caries" tends to oversimplify matters, one cannot discount the essential importance of carbohydrates. However, not too much consideration has been given to the role of other foods whose residues can be conducive to the maintenance and propagation of the pathogenic organisms involved. Is it unreasonable to assume that individual foods or various combinations which favor and support the pathogenic microbiota might have a contributory potential? For example, dairy products are not generally considered conducive to caries, but rather to be more or less protective (Shaw, 1950; Shaw *et al.*, 1959; Bibby, 1961). In view of the frequency and numerous ways these products are used in modern diets, it seems hazardous to assume that such substrates are always innocent bystanders. To some extent this idea can be inferred from the findings of McClure and Folk (1955) in regard to increases in caries activity associated with heat treatment of skim milk powders.

No one would question the nutritional excellence of milk products, but the designation of these products as "protective" in regard to

caries is open to question because of the recognized capacity of such substances to favor the maintenance and growth of bacteria. In many experimental diets, milk products furnish the main source of protein for microorganisms as well as host, and two recent reports of human studies merit thoughtful consideration. Magnusson (1962) has reported that the saliva of newborn infants remains neutral to weakly alkaline during the first 12 hours of life and becomes increasingly acid during the remainder of the first and second day; on the third day the pH levels off to a range between 5.0 and 5.5. "The gradual increase of acidity of the saliva during the first days of life seems to proceed at the same rate as the bacterial colonization of the oral cavity." In the light of these findings the question raised by Fass (1962), "Is bottle feeding of milk a factor in dental caries?" deserves attention not only for the cases he discusses, but also from the viewpoint of the dietary and eating patterns of older individuals. Because the implications of these observations are of considerable magnitude, it might be desirable to recognize this possibility and to consider whether any measures should be recommended to reduce the potential of a deleterious effect after the consumption of certain other foods not usually considered to be cariogenic and high in carbohydrate.

The foregoing remarks are intended to point out that evaluations of the cariogenic potential of foodstuffs are exceedingly difficult because of the conditions illustrated in Fig. 3, and there would seem to be a number of reservations and limitations to information obtained from both *in vivo* and *in vitro* systems which attempt to define the role of monosaccharides, disaccharides, adhesiveness, and the like.

Microflora

Generally speaking, caries has been regarded more as a nonspecific disease than as a specific one (MacDonald, 1962). Numerous acidogenic organisms have been implicated, with most attention directed toward lactobacilli and streptococci. In gnotobiotic studies with rats, certain strains of enterococci and streptococci have shown an unquestionable cariogenic potential (Orland *et al.*, 1955; Fitzgerald

et al., 1960). In his studies to date, Fitzgerald (1962) has not found caries in rats monoinfected with rat strains of *Lactobacillus fermenti*, *Lactobacillus acidophilus*, *Streptococcus lactis*, and *Streptococcus fecalis* var. *zymogenes*.

It was previously demonstrated that after the cariogenic flora of animals had been depressed with an antibiotic, their caries experience was markedly less than in nondepressed controls or in reinfected littermates (Keyes, 1960*a*; Keyes and Fitzgerald, 1963). See Fig. 4.

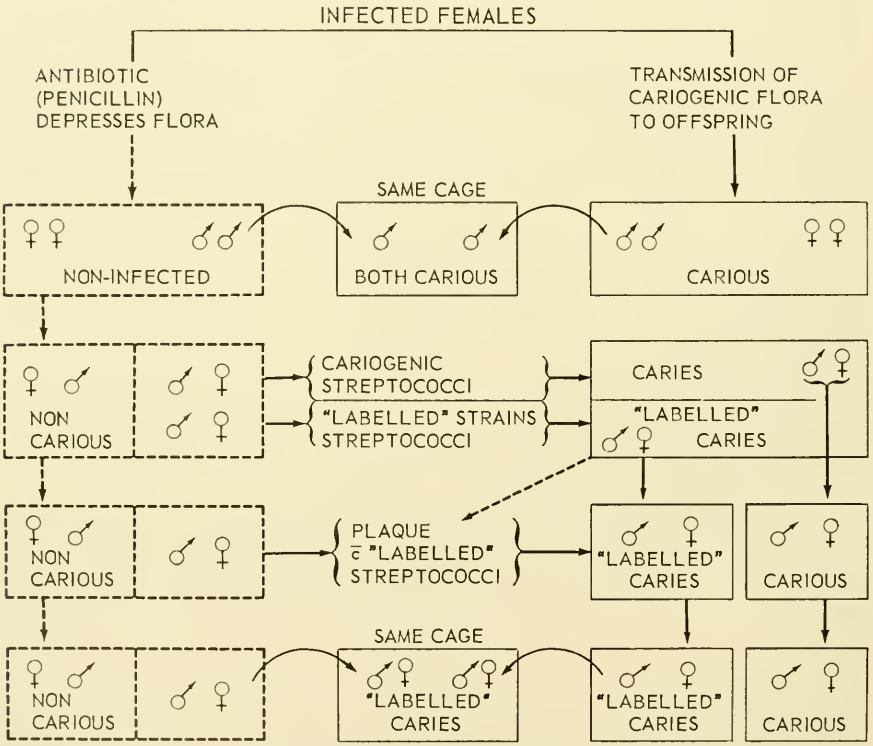

FIG. 4. In the schematic diagram above, on the left are represented several generations of hamsters which were caries-inactive as a result of depression of the cariogenic microbiota. The infection has been reintroduced by contact with infected animals, by an inoculation of both original strains of streptococci and "labeled" streptomycin-resistant strains, and also by the transfer of cariogenic plaque. Following reintroduction of the cariogenic microbiota, caries is again transmitted by natural passage as indicated by solid lines on the right.

Other findings in coincidence with these include those of Zipkin *et al.* (1960) and of Larson and Zipkin (1961).

By the use of bacterially depressed or similar noninfected animals it is possible to show that caries is related to an infectious agent which can be passed from cagemate to cagemate and from mother to young. The disease can also be induced by inoculating either feces or carious dentin and plaque into the mouth of a noninfected young animal. The point has been emphasized that because the alimentary canal microbiota with its cariogenic components is not stable but varies with different experimental diets and conditions, it is difficult to design and interpret many caries studies in animals. For example, many attempts have been made to correlate caries susceptibility with prenatal diets and those consumed during the early stages of tooth formation. The mechanisms whereby protein-carbohydrate ratios, ash and salt mixtures, dietary deficiencies, etc., influence susceptibility of the host cannot be determined if the diet induces unrecognized changes in the alimentary canal microflora of mother animals, who then transmit their altered flora to the young.

It has also been pointed out that in hamsters the dietary history of the mother and her own caries experience may be independent of the susceptibility to caries of her offspring (Keyes, 1960*b*). A susceptible and *infected* female will not develop caries unless maintained on a cariogenic diet; but since she harbors a pathogenic flora, her offspring will develop caries if a suitable diet is provided while the animals are young. It has also been reported (Keyes and Fitzgerald, 1962) that a female can harbor cariogenic bacteria and consume a caries-conducive ration, and still be caries-inactive for various reasons, e.g. maturation factors, etc. Yet such an animal can transmit the flora to her offspring, who will develop the disease. Since the entire alimentary canal microbiota is involved and not the mouth alone, one would not expect to find offspring from females whose teeth had been extracted to be appreciably different from animals whose dams were allowed to develop the disease in retained teeth. This possibility, as postulated by Shaw *et al.* (1962), would seem unlikely. Indeed, animals in which caries activity has been suppressed by fluoride continue to harbor pathogenic organisms and to produce infected offspring (Keyes *et al.*, 1962), and even though the

combination of penicillin and a concomitant change in diet will abruptly arrest caries in the teeth, it does not always depress the cariogenic microbiota so completely that offspring are caries-inactive when tested (Keyes and Fitzgerald, 1963).

The implications of experimental caries transmissibility have not been readily understood. Probably the most important implication of caries transmission is that conventional laboratory animals, notably hamsters, can be obtained which harbor a negligible number of cariogenic microorganisms. Such animals can be used in the design of a "normal" experimental model for studying biological interactions associated with caries. See Fig. 4. The procedure involves less complicated technics than the gnotobiotic ones, and the methods are within reach of almost any laboratory. Investigators have a method for studying caries in conventional animals which is not basically different from those used in the studies of other experimental infections, e.g. tuberculosis (Ratcliffe and Palladino, 1953).

Many attempts have been made to induce experimental caries by an inoculation of organisms thought to have a cariogenic potential (Etchells and Devereux, 1932–1933; Rosebury *et al.*, 1934; Belding and Belding, 1943; and others). However, unequivocal results were not obtained before the use of the previously discussed gnotobiotic technics (Orland *et al.*, 1955; Fitzgerald *et al.*, 1960).

It has been interesting to find that dental caries can be induced by an inoculation of pure cultures of certain organisms isolated from the mouth of caries-active hamsters (Fitzgerald and Keyes, 1960, 1963). Rampant coronal caries has only followed the inoculation of specific strains of hamster streptococci in association with the proper diet. See Fig. 5. It has *not* followed the inoculation of various other strains of acidogenic streptococci isolated from the mouths of either caries-free hamsters or caries-active rats, nor the inoculation of lactobacilli and numerous other acidogenic and non-acidogenic organisms; i.e., coronal caries has not been induced by inoculations of 15 strains of fecal and oral streptococci, lactobacilli, and diphtheroids, and 5 strains of acidogenic filaments, sarcinas, fusiforms, and Gram-negative rods, all strains indigenous to the hamster (Fitzgerald and Keyes, 1960, 1963). Although cariogenic streptococci seem to have a proclivity for forming plaque, it should be emphasized that not all

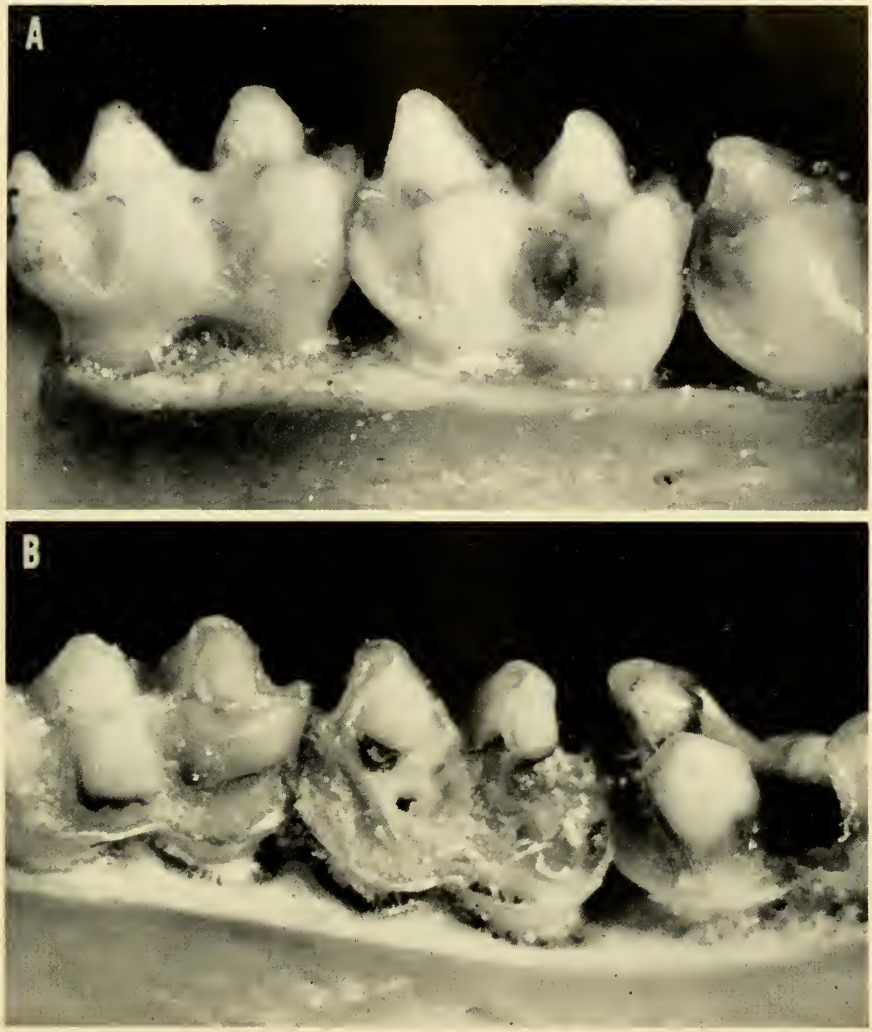

FIG. 5. A, no cavitation has occurred in teeth in control animal because it did not harbor cariogenic bacteria during the early stages of its life. B, extensive caries followed the inoculation of cariogenic streptococci isolated from a carious lesion in another hamster.

plaques found on hamster teeth have been associated with typical caries. Apparently plaques do not form unless specific types of organisms are present.

In hamsters, cariogenic plaque formation does not appear to be simply the result of bacterial growth in food residues coincidently retained on the teeth, although in certain instances this may be the case. The plaque which so rapidly forms in either naturally infected or inoculated animals appears to be the product of an interaction between bacteria and the diet. There seem to be specific conditions which determine whether colonization occurs or not. This is to say that, experimentally, the formation of the plaque itself is not the result of one set of factors but of the simultaneous interaction of two sets, or three if one adds those contributed by the enamel.

Question of Bacterial Specificity

It seems important to clarify the etiologic role of the acidogenic microbiota in caries initiation *in vivo*. On the basis of what is generally recognized about the specific nature of the biochemical, nutritional, and environmental factors which determine implantation and propagation of microorganisms, it does not seem unreasonable to postulate a degree of specificity to caries initiation. This does not imply that every lesion would have exactly the same microbiological component; indeed, some observations in rats and hamsters suggest that this may not be so, but probably the point will require gnotobiotic technics to determine conclusively. Nevertheless, the position of streptococci in an environment with high concentrations of carbohydrate would seem to be more delicate than that of lactobacilli. The fact that streptococci are not so aciduric suggests that these organisms would thrive better in a locus in which their own acid by-products could be readily neutralized, as well as those from other neighboring organisms.

A question not too frequently asked is, "What does the carious lesion, i.e. the invasion of the enamel and dentin, mean to the organisms involved?" Certainly from numerous histopathological studies caries appears to be a highly invasive infection. Figure 6 shows several examples of bacterial invasion in hamster molars. Is this phe-

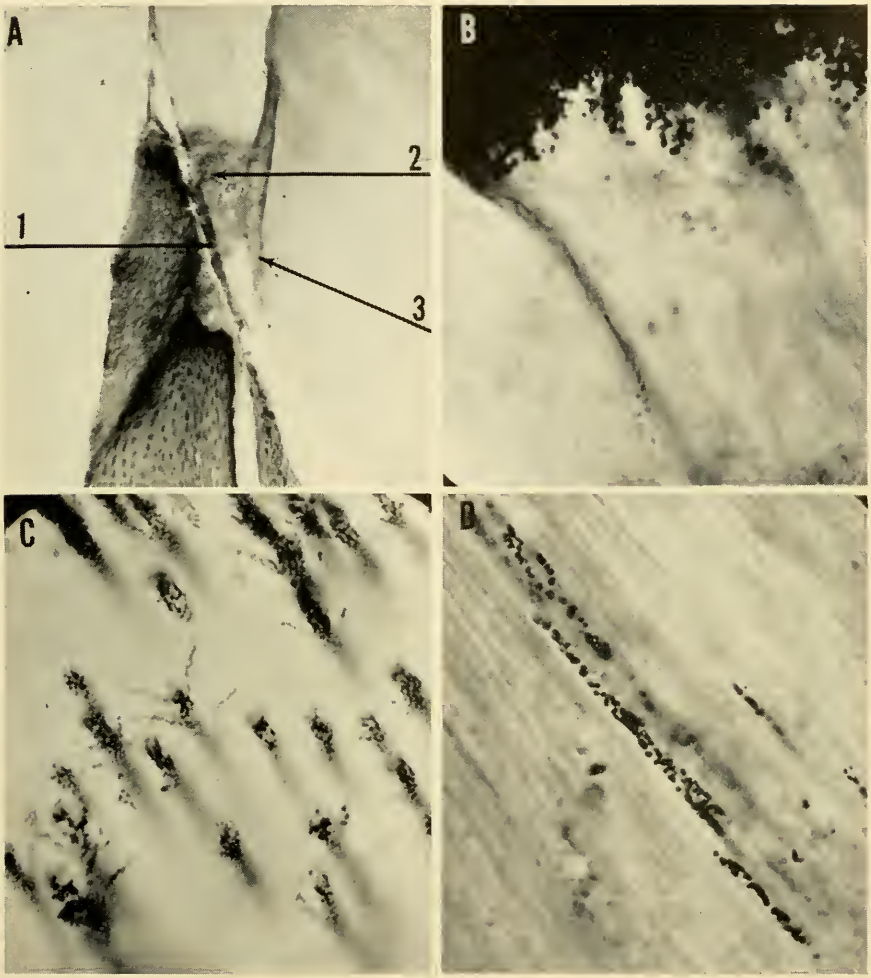

FIG. 6. Decalcified sections of carious lesions in the hamster. A, plaque (1) formation in the gingival sulcus associated with massive invasion of enamel (2); and characteristic spread along the junction of enamel and dentin (3). B, Gram-positive spherical organisms invading enamel. C, cross sections of dentinal tubules filled with Gram-positive cocci. D, tubular invasion often can be followed for considerable distances in the dentin. Only coccoid forms of organisms have been found in the depths of lesions, approaching the pulp, and far in advance of generalized necrosis. Photographs B, C, and D were taken under oil immersion.

nomenon merely accidental or coincidental? Orland (1946) noted that streptococci were found in great numbers in hamsters with highly active caries. Although he recovered many lactobacilli, among other bacterial types, he mentioned that the streptococci might be less aciduric than lactobacilli, and went on to speculate that the source of these bacteria might be in the less acid environment of deeper cavities. Harrison (1948) stated: "It is presumed that bacteria may cause dissolution of one or more components of the enamel structure, either to secure their own metabolic requirements or as an incidental or accidental consequence of the release of harmful metabolic products on the enamel surface."

In the hamster, plaques develop on coronal surfaces of the teeth and in the gingival sulcus when certain dietary substrates are available and specific types of bacteria are harbored in the alimentary canal. As previously stated, neither one of these factors by itself appears adequate to induce the phenomenon.

> It cannot be too strongly emphasized that none of those regions of the body, to which bacteria can gain entrance by the normal portals, provides a virgin soil in which any newcomer may flourish. It is just as impossible to ensure the proliferation of a particular bacterial species by introducing it into the mouth, as it is to ensure a crop of a particular plant by scattering seeds in a field already occupied by a pre-existing plant-association. The newcomer will have little chance of survival, unless it is adapted to occupy some definite place in its new environment. It is fairly certain that those pathogenic bacteria which spread readily from host to host owe their capacities in this direction to their ability to colonize on skin or mucous surfaces, or to escape from the superficial environment to the underlying tissues. (Wilson and Miles, 1955.)

The findings of Fitzgerald and Keyes (1960) have indicated that many types of acidogenic bacteria normally found in the mouths of hamsters do not cause plaque formation even when a high-carbohydrate low-fat diet passes through the mouth and alimentary canal; i.e., the teeth generally remain remarkably free of these bacterial accumulations. When colonization of cariogenic microorganisms does occur and when the tooth is in a susceptible state (newly erupted and low in fluoride content), demineralization and invasion rapidly occur (Keyes, 1959). The findings of Fitzgerald and Keyes (1960)

have been misconstrued by Schatz and Martin (1962): "If decay were due to acid, all the aforementioned organisms should have been cariogenic since all are acidogenic." The reasoning here is open to question because in the experimental hamster model not all acidogenic organisms will colonize and produce plaques on enamel surfaces. The results of Fitzgerald and Keyes (1960) should not be used to support the proteolysis-chelation theory.

The discussion of streptococci by Burnett and Scherp (1962) is quoted below because many observations on caries activity seem to be in coincidence with what is known about this group of bacteria:

> The environmental and nutritional requirements of streptococci differ widely among various strains and species, ranging from the most exacting conditions to the ability to grow in a very adverse environment. The human pathogenic strains are generally more exacting in their environmental and nutritional requirements than are the nonpathogenic strains. The following essentials have been recognized: Carbohydrates—a readily available source of carbohydrate is necessary for the growth of aerobic streptococci, although it may not be essential for the growth of some species of anaerobic streptococci. The amount of carbohydrate available in media may be critical; if too much is available, sufficient acid is produced to inhibit or kill streptococci; if not enough is present, growth does not occur. Carbon dioxide—a proper amount of carbon dioxide (usually 5 per cent) favors growth and utilization of nutritive material and may act, for instance, as a substitute for purines. Vitamins—some water-soluble vitamins such as biotin, pantothenic and nicotinic acid, thiamine, riboflavin, pyridoxal, and folic acid are essential for growth, varying with the species. Purines, pyrimidines, and as yet undefined growth-promoting substances, e.g., yeast or liver extracts, may enhance or even be essential for the growth of the more fastidious streptococci. Inorganic salts and buffers—although a balanced inorganic ion system is needed for the growth of streptococci, their specific requirements have not been adequately defined. Good buffers are necessary for the neutralization of acids produced during metabolism.

Questions about the requirements for CO_2 and for inorganic salts and buffers have been of interest to us. Is there a special need when certain organisms have to deal with high loads of carbohydrates? It seems reasonable to postulate that those organisms which have invaded the tooth are surviving and probably finding a favorable

environment. If inorganic ions form part of this milieu, which ones and why?

There are many interesting questions related to the specific requirements of microorganisms and how they invade. Along these lines, the mechanisms whereby fluoride exerts its protective effect might again be examined in terms of what the inability to invade might mean to the organisms involved. The two effects most often advanced to explain the action of fluoride are a decreased solubility of the apatite mineral and an antienzymatic effect which inhibits glycolysis. Recent observations in hamsters have shown that caries can be effectively inhibited by adding fluoride to the drinking water at the time of third molar eruption, i.e., during the very early eruptive and posteruptive period. Although lesions which started before the addition of the fluoride ion continued to progress slowly and known cariogenic streptococci were recovered from the mouth in high numbers (Keyes *et al.*, 1962), the third molars were exceedingly well protected. Also, this tooth acquired a long-lasting resistance, as indicated by its ability to remain intact long after withdrawal of the drug.

Apparently the colonization of pathogenic organisms was not prevented sufficiently to explain the lowered level of caries activity. It would be of interest to know whether the organisms were behaving as usual. Is it possible that the reduced solubility of the apatite (Gray *et al.*, 1962; Zipkin *et al.*, 1962) and its altered chemical composition (Brudevold, 1962*a*, 1962*b*; Nikiforuk *et al.*, 1962) prevent the pathogenic bacteria from obtaining essential requirements at a critical time? For example, the amount of salivary CO_2 available at the interface between cell membrane and crystal and deep in the lesion is unknown, but it may not be enough for organisms faced with the problem of rapid metabolic activity, especially since salivary CO_2 probably does not diffuse readily through the acid plaque and lesion. The reduced solubility of enamel mineral and replacement of hydroxyl ions could conceivably reduce the availability of buffers necessary for neutralization of the acids produced during metabolism.

One is able to think of many possibilities in regard to what the effect of a change in the availability of inorganic ions can mean to

bacteria which are confronted with the metabolism of copious amounts of carbohydrates, often in the form of hypertonic sugar solutions. Perhaps the organisms have little choice other than rapid metabolism, accelerated reproduction, or polysaccharide storage (Gibbons and Socransky, 1962). (The latter phenomenon may also represent a protective mechanism whereby less aciduric organisms can delay the conversion of large quantities of carbohydrate and thus control the release of unfavorable amounts of free acid. Polysaccharide storage apparently immobilizes part of the carbohydrate for future metabolization at a gradual rate.)

We have found these questions intriguing and have found it interesting to speculate on possible mechanisms which might affect bacterial activity from what is known about the inorganic ion requirements of streptococci, changes in the carbonate content of enamel containing fluoride (Brudevold, 1962*a;* Nikiforuk *et al.,* 1962; Middleton, 1962), and the loss of carbonate and magnesium from carious enamel and dentin (Johansen, 1962).

Along these lines the report of Brown *et al.* (1962) merits comment. These workers postulated that essential nutrients diffusing from within the tooth might support growth of invading microorganisms and thus play a role in caries. In an *in vitro* system of special design it was possible to demonstrate that organisms penetrated dentinal tubules when the crowns of teeth were covered with a niacin-deficient medium, and the growth of *Lactobacillus arabinosus* depended on the diffusion of niacin from the pulp side of the teeth. The principles of this system and the implications of the findings are of considerable interest, as are the original demonstrations. Invasion seems to be related to dependence of organisms upon essential factors.

Clinical and Therapeutic Implications

The matters discussed have a bearing on the clinical disease. We do not know whether human caries is largely a specific type of microbial infection, particularly in the initial stages. The etiologic role lactobacilli and other acidogenic organisms play in human caries needs further clarification. On the basis of such findings im-

proved diagnostic tests might be developed. If specific organisms occupy a key position in caries initiation, chemotherapeutic agents would have a greater potential in anticaries therapy. Contagiousness and transmissibility of the cariogenic agent (streptococci or otherwise) are of more concern to laboratory workers, who need to balance animals microbiologically in order to control their experiments and who need to determine the bacterial conditions in the mouth and intestinal tract in order to interpret their results.

The next few years should see the development of more effective programs for caries control which would employ a combination of therapeutic measures based on the considerable amount of information available. It is doubtful, human nature being what it is, that any single measure will be entirely effective in preventing and arresting this disease. Changes in diets and the avoidance of unfavorable eating habits are to be strongly advocated (Bibby, 1962; Nizel, 1962; and others). The consumption of fluoridated water is of unquestionable benefit, and the supplemental use of fluorides appears to be indicated on theoretical grounds. Further work on other modes of fluoride application is certainly needed. Antibacterial drugs have occasionally been used in anticaries therapy for almost four decades (Bunting *et al.*, 1927; Strålfors, 1962); but few, if any, have been thoroughly tested in a comprehensive program of treatment. In the treatment of this infection, with its highly invasive characteristics, it will be necessary to employ the proper type of drug and to devise methods of application which not only will ensure its presence in susceptible areas but also will keep it in sufficient concentration and for adequate periods of time.

Summary

Dental caries activity is determined by interactions in a factorial triad comprising the host, the diet, and the microflora. As yet only specific strains of streptococci have induced typical coronal caries in noninfected hamsters and in more rigidly controlled germ-free (gnotobiotic) rats. In hamsters kept under conventional laboratory conditions, lactobacilli and various other acidogenic organisms, including other strains of streptococci, have not induced highly active

disease. The demonstration of caries infectivity in hamsters shows investigators how to produce quasi gnotobiotes which can be used for more refined experimental models to study some of the biomechanisms involved. These observations raise the question whether caries is a more specific type of bacterial infection than is usually supposed. It certainly appears that all acidogenic organisms are not equally cariogenic. Some lesions might follow the activity of organisms which are adapted to use the tooth as a source of beneficial substrate, especially when faced with high levels of carbohydrates in their immediate environment. Further leads on diagnosis, causation, and control could conceivably follow research directed toward the disclosure of other bacterial characteristics besides acid production, e.g. the nature of the organic components of the cells, their cell walls, and extracellular products. Fully effective caries control can be achieved under laboratory conditions. Probably equally effective control can be attained in humans by comprehensive therapeutic measures analogous to those used in the treatment of tuberculosis, i.e. improvement in eating habits, increasing resistance of tissues (enamel), chemotherapeutic depression of pathogenic organisms, and necessary operative (surgical) procedures.

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10

Effect of Hibernation on Tooth Structure and Dental Caries

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AT A TIME when the public press, popular science writers, and even some professional scientists have speculated on hibernation as a mechanism whereby humans could travel great distances through space in the state of torpor accompanying the phenomenon of hibernation, it is strange that we have so little evidence of the effect of hibernation on the morphology and physiology of mammals. The studies of Mayer (1960) and Mayer and Bernick have dealt with aspects of hibernation in relation to the structure and function of endocrine glands (1959), the digestive system (1958), and protein and carbohydrate metabolism (1957). Almost nothing, however, is known of the effect of hibernation on bones and teeth.

Sarnat and Hook, in 1942, concluded that all stages of tooth development, including growth, calcification, and eruption, were severely retarded in proportion to the time the animal was in hibernation. However, they made no detailed histological studies of the effects of hibernation on tooth and bone development. The study of Richardson *et al.* (1961) did not provide unequivocal evidence regarding the effect of hibernation on the dental tissues of the 13-

lined ground squirrel. In the present investigation the authors have examined histologically the effects of hibernation on the teeth and on the surrounding bones and periodontium of a hibernating animal.

Hibernation is a relatively rare phenomenon. The term is correctly used to identify that metabolic state involving cessation of externally observable activity and a marked lowering of the body temperature of the homoiotherm. Though it is commonly thought of as a phenomenon of the high Arctic, it is in reality a temperate-zone phenomenon, to judge from the geographic distribution of the animals which actually hibernate. The phenomenon is limited to relatively few genera of birds and mammals and is considered by the senior author to be a highly stressful and relatively unsatisfactory method of meeting environmental extremes, at least for the individual animal, although it is sufficiently effective for species survival.

The selection of the Arctic ground squirrel as the hibernator for use in these experiments is based on the facts that it is a large animal readily kept in captivity and that it can be induced to hibernate relatively easily. Mayer (1953*a*, 1953*b*) has reported on the ecological relationships of the Arctic ground squirrel and its patterns of hibernation. Although in this paper such terms as "3 months in hibernation" are used, it is to be understood that hibernation is not a continuous process, operating from September until May in the case of the Arctic ground squirrel, but rather a series of hibernations from which the animal periodically awakens at roughly 3-week intervals. Thus, a 3-month period of hibernation would undoubtedly have included at least four such awakenings.

The Arctic ground squirrels for this study were obtained from Alaska and maintained in colonies in our laboratories.

Materials and Methods

The animals used for this study were Arctic ground squirrels (*Citellus undulatus*), ranging in age from 3 to 4½ years. In captivity they had been subsisting on a natural stock ration (Purina) supplemented with vegetables, such as lettuce, cabbage, carrots, or celery.

Animals were sacrificed and the skulls removed, decalcified, em-

bedded in celloidin, sectioned, and prepared for examination histologically in the routine manner.

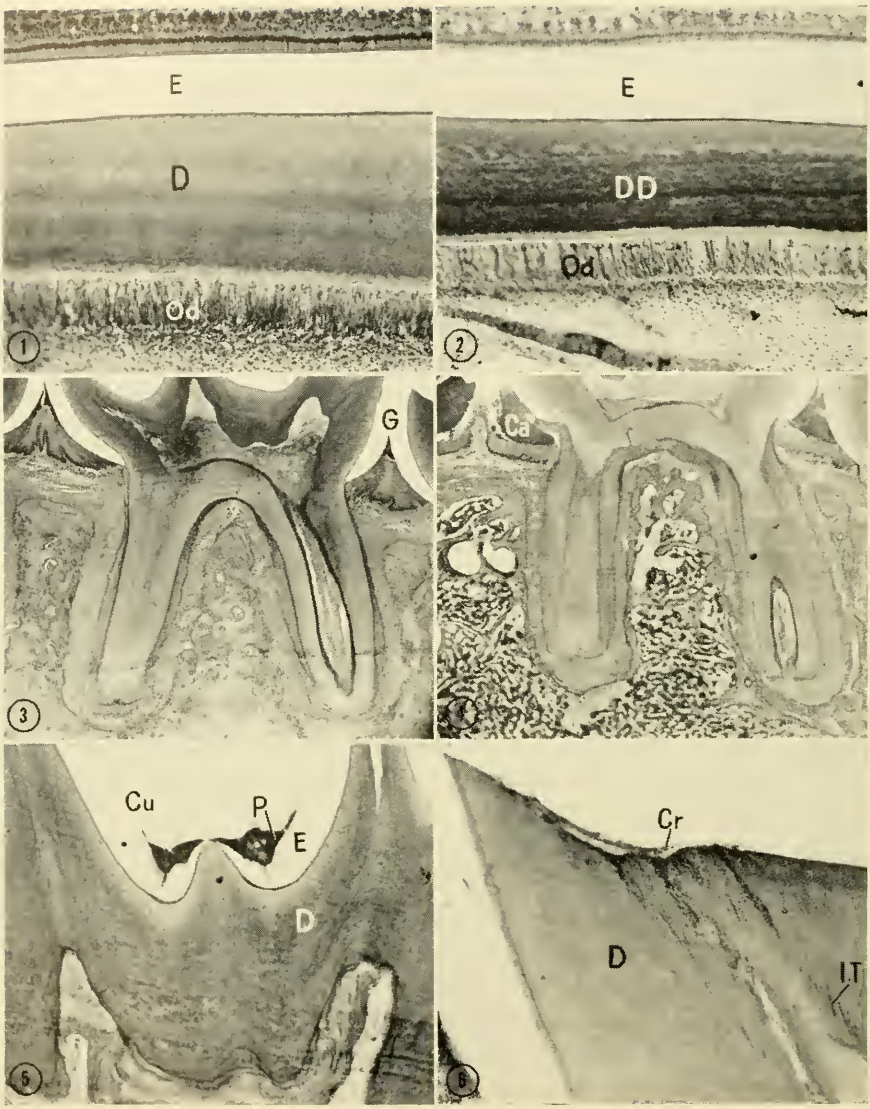
Observations

The dentin of a nonhibernating Arctic ground squirrel presents a wide, homogeneously calcified outer layer which extends from its outer surface through the uncalcified predentin toward the odontoblastic layer (Fig. 1). After about 3 weeks in hibernation, however, there is evidence of a disturbed calcification process (Fig. 2). One may still see a thinner outer zone of dentin which is homogeneously calcified. There is, however, an increase in the number of interglobular spaces in the deficient dentin extending toward the odontoblastic layer.

In nonhibernating Arctic ground squirrels the interradicular and the interseptal bone show a high degree of calcification and both are compact and normal in histological configuration (Fig. 3). In contrast, after an animal has been in hibernation for 3 months there is a progressive loss or resorption of both interradicular and interseptal bone. Figure 4 shows a marked osteoporosis of the spongiosa and the presence of calculus in the interproximal gingivae. Even though the alveolar bone proper and the attachment fibers are relatively unaffected in this animal, alveolar bone frequently undergoes degenerative changes during hibernation.

Caries appears rather consistently in both the molar and premolar teeth of squirrels kept in captivity for extended periods of time. As field-caught wild animals do not show such a predisposition to caries, it is believed that decay is not related so much to hibernation as to conditions of captivity, including diet. However, as the effects of hibernation on noncarious normal teeth are drastic, in carious teeth these effects even further aggravate tooth destruction.

All stages of caries, from early lesions to fracturing of the coronal part of the tooth, accompany hibernation. Figure 5 shows early carious lesions. Two plaques occur in the occlusal pits, and from these plaques dark-staining lamellae project through the decalcified enamel toward the dentin. Figure 6 shows a more advanced carious lesion which involves an invasion of the dentinal tubules. The in-



FIGURES 1 TO 6

fected tubules extend from the surface lesion deep into the substance of the dentin. In Fig. 7, a further aggravated lesion, in which the dentin has been locally decalcified, has produced a typical carious cavity containing a heavily stained plaque.

Pulpal involvement is of common occurrence as demonstrated in Fig. 8, which represents a buccal-lingual section of the upper first molar. The carious lesion has invaded the pulpal area, and the process of degenerative change has resulted in an open communication with the oral cavity. The disintegration of the pulp extends into the root to produce an apical abscess. The inflammatory lesion has infiltrated the interradicular region and has extended to the adjacent bone. In this figure the periapical abscess is pronounced.

In an animal in hibernation for 3 months, a buccal-lingual section of an upper molar (Fig. 9) demonstrates open communication of the pulp with the oral cavity. In this case, the destruction of the

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Abbreviations Used in Figures

A, abscess
B, bone
C, cementum
Ca, calculus
CE, cemento-enamel junction
Cr, carious lesion
Cu, cuticle
D, dentin
DD, deficient dentin
E, enamel
EA, epithelial attachment
Ep, epithelium
G, gingiva
IT, infected tubules
Od, odontoblastic layer
P, plaque
PA, pulpal abscess
PP, periodontal pocket
Pu, pulp
R, root
RF, root fragment

FIG. 1. Incisal dentin of a warm and active Arctic ground squirrel. The dentin is homogeneously calcified.

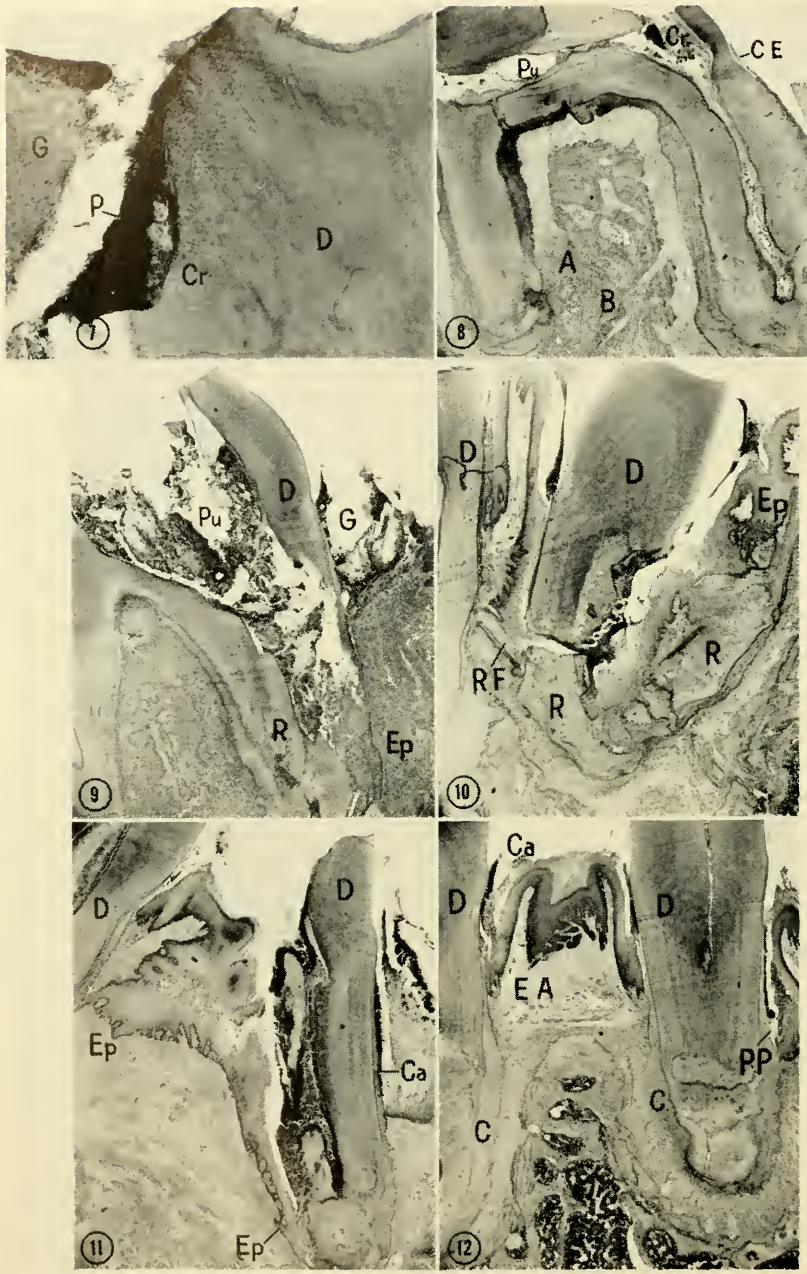
FIG. 2. Incisal dentin of squirrel in hibernation for 3 weeks. The deficient dentin shows an increased number of interglobular spaces.

FIG. 3. Upper molar of a warm and active squirrel. The compactness of the interseptal and interradicular bone is normal.

FIG. 4. Upper molar of an animal in hibernation for 3 months. Osteoporosis of the spongiosa of both the interseptal and the interradicular bones is obvious. Calculus is present in the interproximal gingivae.

FIG. 5. Upper molar of an animal in hibernation for 3 weeks. From the plaques in the occlusal pits heavily stained lamellae project toward the dentin.

FIG. 6. Upper molar of an animal in hibernation for 3 weeks. The dentinal tubules have become infected by carious invasion.



FIGURES 7 TO 12

pulp has extended down into the root of the tooth. This pulpal inflammation has hollowed out the root canal, resulting in a thinning of the root while the necrotic pulp fills with debris. In addition, the epithelial attachment has proliferated deeply along the sides of the root toward the apex, with extensive destruction of the gingiva on the buccal face of the molar.

In another animal in hibernation for 3 months, the pulpal involvement has become so extensive as to lead to a fracturing of the coronal part of the tooth (Fig. 10). The epithelium has invaded the base of the fracture, and both the surface epithelium and the inflammatory cells have proliferated deeply to the apex of the tooth fragments. One fractured fragment has been encircled by epithelium.

The encircling of fragments by epithelium takes place at various times during hibernation, as shown in Fig. 11, which is a section of a premolar from an animal in hibernation for 1 month. In this case, a persistent fragment of the tooth is encapsulated by epithelium. Pus cells are seen adjacent to the open end of the tooth, and the epithelium has proliferated to the apical end of the fragment, forming a deep periodontal pocket on the distal surface of the tooth fragment. The mesial fragment from this tooth has been exfoliated, and a periodontal pocket is observed on its distal surface.

FIG. 7. Upper molar of an animal in hibernation for 1 month. The carious cavity in the locally decalcified dentin is extensive.

FIG. 8. A buccal-lingual section of an upper molar of an animal in hibernation for 3 weeks. The carious lesion has invaded the pulp, resulting in its open communication with the oral cavity. The periapical abscess has invaded the adjacent bone.

FIG. 9. A buccal-lingual section of an upper molar of an animal in hibernation for 3 months. The destruction of the pulp has extended deep into the roots, resulting in a thinning of the root. Downgrowth of epithelium toward the apex and destruction of the gingiva on the buccal surface have also occurred.

FIG. 10. A premolar of an animal in hibernation for 3 months. The coronal part of the tooth has fractured, and the epithelium has invaded the base of the fracture.

FIG. 11. A premolar of an animal in hibernation for 1 month. A persistent fragment of the tooth is encapsulated by epithelium. The deep periodontal pocket on the distal surface of the tooth fragment is indicative of extensive degeneration.

FIG. 12. The interproximal region between two premolars of an animal in hibernation for 3 weeks. Pocket formation and the presence of calculus on all surfaces is obvious.

In addition to the effects of hibernation on caries and its effects on bone and dentinogenesis, animals in hibernation for periods of from 3 weeks to 3 months exhibit various degrees of periodontal involvement. Osteoporosis of bone was shown in Fig. 4 in an animal which had been hibernating for 3 months. Figure 12 represents the interproximal region of two premolars of an animal hibernating for 3 weeks. There is a loss of trabeculation, although less than that observed in Fig. 4. There is, however, a pocket formation on both the mesial and distal surfaces of the two adjacent teeth, and the distal surface of the second premolar exhibits a deep pocket that extends to the lower half of the root. In addition, the presence of calculus is obvious. In still another hibernating squirrel the distal region of the first upper molar exhibits a degeneration of the surface epithelium and the presence of calculus on the cementum (Fig. 13). There is also a noticeable loss of interseptal bone and a further downgrowth of the epithelial attachment.

After 3 months in hibernation a section of the interproximal region between two molars of a squirrel shows an epithelial attachment which has proliferated to the apex of the root (Fig. 14). The crest of the bone has been lowered to the apical region. The cemental

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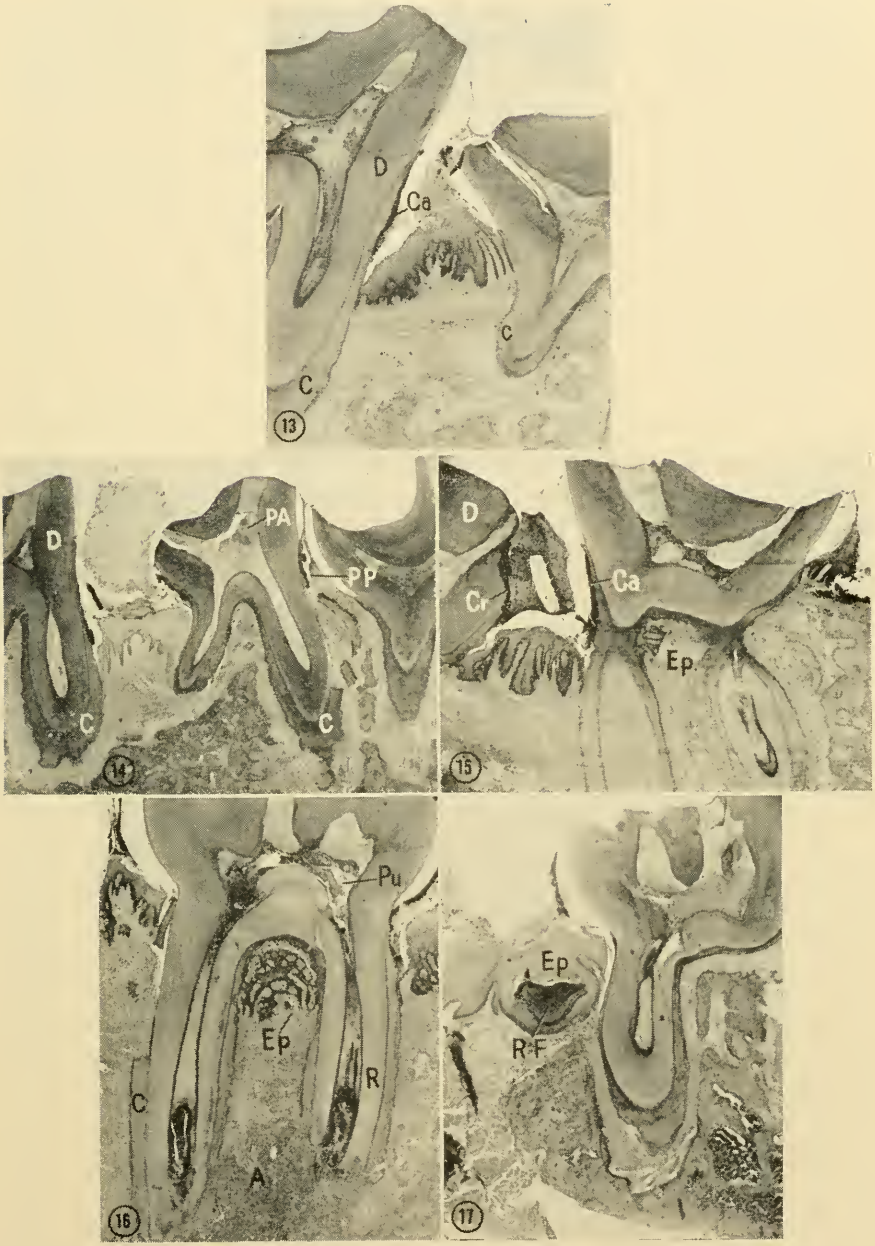
Fig. 13. Interproximal region between two molars of a hibernating animal. Further downgrowth of the epithelial attachment and loss of interseptal bone are common during hibernation.

Fig. 14. Interproximal region between two molars of an animal in hibernation. The epithelial attachment has proliferated to the apex of the root. The crest of the bone has been lowered to the apical region. Cemental tears on the distal surface of the molariform tooth, a periodontal pocket in the gingiva of the same surface, and the pulpal abscess indicate the deleterious effects of hibernation on teeth.

Fig. 15. Interproximal region between two molars of an animal in hibernation. The presence of epithelium in the interradicular region, the lowering of the crest of the bone, and the carious lesion in the dentin further attest to the stresses of hibernation.

Fig. 16. Upper first molar of an animal in hibernation for 3 months. The bifurcation involvement with loss of attachment fibers and bone, the periapical abscess, and the cemental tears indicate further deterioration in the oral cavity during hibernation.

Fig. 17. Upper first molar of a hibernating animal. The complete encapsulation of a root fragment by epithelium is a response to the degeneration of tooth structure during hibernation.



FIGURES 13 TO 17

tears on the distal surface of the molariform tooth are obvious. A periodontal pocket with a proliferating epithelial attachment is present in the gingivae of the same surface. A pulpal abscess is present and its communication with the oral cavity is noticeable.

The interproximal region between two molars from another hibernating squirrel shows a downgrowth of the epithelial attachment, which proliferates into the interradicular area (Fig. 15). The invasion of the epithelium into the bifurcation region is accompanied by a disorganization of the attachment fibers and a lowering of the alveolar bone. This degeneration is accompanied by carious lesions of the dentin.

In Fig. 16, showing a first upper molar of a squirrel in hibernation for 3 months, continued deterioration is demonstrated. Periodontal pockets on the mesial and distal surface exhibit such extensive bifurcation involvement that no bone remains in the interradicular region. In addition, an apical abscess is present and there is an inflammatory invasion of surrounding connective tissue. Epithelial encapsulation of tooth fragments is frequently noted in squirrels in hibernation. Figure 17 shows a root fragment fully encapsulated by epithelium.

There can be no doubt that hibernation has a dramatic effect on bone, dentinogenesis, and dental caries. The reasons why this should be so, however, are not elucidated in the literature. In the work of Willet *et al.* (1957) it was indicated that protease may accelerate caries in several ways. The work of Hunt *et al.* (1957) showed that submaxillary gland extracts of caries-susceptible rats had $5\frac{1}{2}$ times the protease activity of gland extracts from more resistant animals. The work of Mayer and Bernick, however, demonstrated an involution of the salivary glands during hibernation and thus an inhibition of protease activity. Thus, it is not to the salivaries that we can turn for our explanation of aggravated carious lesions during hibernation.

Rosen *et al.* (1957) indicate that it is highly probable that microorganisms are the immediate causes of dental caries. During hibernation, however, there is not only a lowered oral cavity temperature, but also a drier oral environment, due to cessation of salivary activity. Thus, one would be led to anticipate less and not more mi-

crobial activity during hibernation because the factors of moisture and temperature during hibernation are inimical to growth of microorganisms.

Hunt *et al.* (1957) cited work which concluded that endocrine secretions are a factor in dental caries, but here again Mayer and Bernick (1959) have indicated a general involution of the endocrine system during hibernation. Two points in Hunt's paper may point the way to a possible explanation of the extensive dental caries in captive Arctic ground squirrels. One involves dietary factors, which have an evident effect on caries. The other is that a desalivated animal, in which conditions similar to those of an animal in hibernation are simulated, is not able to wash food away from its teeth as well as the animal with active salivary secretions. The resulting prolonged contact of food with teeth, regardless of lowered temperature, may contribute greatly to the accelerated development of caries in hibernating animals.

Summary and Conclusions

The observations reported in this paper underscore the lability of bone minerals during hibernation, when both teeth and bone are called upon to contribute calcium to the metabolism of the hibernator.

Hibernation results in deficient dentinogenesis, a reduction and osteoporosis of both interseptal and interradicular bone, and degeneration of alveolar bone, as well as a downgrowth of surface epithelium, and pocket formation on mesial and distal surfaces of adjacent teeth. There is also a disorganization of attachment fibers and the presence of calculus on the cementum, together with cemental tears. Carious lesions and pulpal and apical abscesses are also aggravated in the teeth of hibernating animals.

The process of hibernation can thus be seen to have severe effects on the metabolism of the hibernating animal. During hibernation there is a severe drain on mineral resources within the body. This drain increases with time and gives one pause in postulating hibernation as an effective mechanism for meeting severe environmental stress, for the process itself is an exceptionally stressful one.

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11

Dento-Alveolar Resorption in Periodontal Disorders

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PERIODONTAL disorders are diseases clinically characterized by (*a*) inflammatory color changes and swelling of the gingiva, (*b*) deepening of the gingival pockets, and sometimes (*c*) increased mobility and migration of the teeth. To some degree, one or more of these symptoms are nearly always found somewhere in the mouths of man in all age groups.

Alveolar bone resorption can be detected very frequently in x-rays of periodontal disorders, and tooth resorption may also occur.

In periodontal disease, pathological changes in the gingiva always are seen at the microscopic level. This is the case even if the typical clinical signs of a gingivitis are missing. Microscopic sections of a human periodontium without inflammatory reactions are rare indeed.

Many theories have been proposed regarding the etiology of bone and tooth resorption in periodontal disorders. These theories will be discussed in this chapter.

Theories Regarding Alveolar Bone Destruction

It has been suggested that alveolar bone destruction is a result of a gingival inflammation (James and Counsell, 1928; Lang, 1923;

Häupl, 1925; Häupl and Lang, 1927), and that it is caused by increased masticatory functional stress (Karolyi, 1905) or by a dystrophy not necessarily related to gingivitis (Weski, 1921, 1928). According to Gottlieb (1925), alveolar bone resorption can be due to an atrophy, or to a gingival inflammation caused by dental calculus or tartar and retention of food ("Schmutzpyorrhoe"). Some authors, among them Thoma and Goldman (1937), have attributed the breakdown of alveolar bone to local (paradontitis) and systemic (paradontosis) factors and to combinations of the two factors. Glickman (1949, 1952) is of the opinion that both local and systemic factors may be responsible for the alveolar bone destruction, the outcome being governed by a so-called "bone factor," defined as the systemic regulatory influence upon the alveolar bone. The last theory to be mentioned, as advanced by Lang (1923) and by Häupl and Lang (1927), differs in many respects from the others, and necessitates an explanation in greater detail of its main points, as follows.

Häupl and Lang, in accordance with the general concept of Pommer (1885, 1925), take the position that circulatory disturbances which increase the tissue tonus in the bone cause bone resorption in all parts of the skeleton. They maintain more specifically that circulatory disturbances of this type develop in the alveolar bone through an interaction of factors which can be divided into three groups: (1) external factors such as irritants initiating a gingival inflammation, and circulatory disturbances in the periodontium caused by mechanical functional stress above the physiological limit; (2) inherited and acquired anatomical and physiological periodontal conditions which directly promote the development of or aggravate disturbances in the blood circulation in the alveolar bone; and (3) inherited and acquired systemic factors which indirectly influence the periodontal blood circulation, e.g., the kinds of influences that are involved in the over-all parasite-host relation of the organism.

Häupl and Lang assert that the loss of alveolar bone in periodontal disorders is determined by the relation between resorption and formation of alveolar bone. Furthermore, they are of the opinion that

formation of alveolar bone may be due to three factors: inherited characteristics, substances produced in the inflamed periodontal tissues, and masticatory functional impulses.

The latter concept is based on the fact that weakening of the alveolar bone as a result of resorption exposes the rest of it to increased functional stress, or what is called hyperfunction. In all parts of the skeleton, hyperfunction to a certain limit results in compensatory bone apposition, provided that the bone tissue has a sufficient supply of hormones, vitamins, calcium salts, and other nutritional elements necessary for bone production. In a growing organism compensatory bone apposition and hereditary bone growth take place at the same time. In this period heredity governs the gross pattern of bone formation, while functional impulses modify the form and structure of the bones.

The stimulating effect of mechanical functional impulses on bone production was established by Roux in 1895, and his findings have been confirmed by clinical observations and a series of experiments, of which only those performed by Häupl and Psansky (1938) and by Watt and Williams (1951) will be mentioned here. The former authors observed that alveolar bone and connective tissue in the pericementum had been produced in humans as a reaction to an increase in masticatory functional stress for three nights by means of activators (Fig. 6). Watt and Williams found that the size and density of the mandible in growing and adult rats increased with a rise in the masticatory functional stress. Both experiments support the assumption that alveolar bone resorption is a trigger mechanism which, subject to certain conditions, incites alveolar bone production.

Instead of any further comment on the different theories, the problems pertinent to the loss of alveolar bone in periodontal disorders will be discussed from the standpoint of (1) the relation between circulatory disturbances in the periodontium and loss of alveolar bone, (2) the effect of increased functional stress on the alveolar bone, (3) the influence of periodontal and systemic conditions on the loss of alveolar bone, and (4) the effect of periodontal pathology on resorption of the teeth themselves.

The Relation between Circulatory Disturbances in the Periodontium and Loss of Alveolar Bone

Gingival inflammations are the most common cause of circulatory disturbances in the periodontium. An increased amount of blood must be transported to and from the inflamed gingiva through the alveolar bone.

Clinical and x-ray observations indicate that a chronic gingivitis very often is accompanied by a breakdown of alveolar bone. Elimination of a gingivitis through successful treatment usually stops the resorption, and sometimes it is followed by an apposition of alveolar bone visible on x-rays.

In histological sections it can also be seen that alveolar bone resorption is related to periodontal circulatory disturbances. If they are confined to the supra-alveolar connective tissue, a horizontal septum resorption occurs. When the vessels passing through the marginal part of the pericementum are involved, vertical septum resorption takes place.

In cases of gingival inflammation, a breakdown of the central part of the septa frequently is observed on x-rays. This resorption may extend from the margin to the base of the septa, and not seldom the lamina dura of the adjacent teeth is broken down in some places (Figs. 1 and 9). According to Häupl and Lang (1927), these resorptions are due to a remote effect of a gingivitis. When an increased amount of blood is transported to an inflamed gingiva through the interalveolar arteries, they will be dilated and compress the veins in the alveolar bone marrow more or less (Fig. 2). The effect of this is an increase of the permeability of the vessels in the septa, and that again enlarges the amount and changes the quality of the transudate to the bone marrow.

In histological sections it can be observed that the alterations in the transudation lead not only to alveolar bone resorption, but also to fibrosis of the alveolar bone marrow. The latter process, called bone phlegmasia according to Recklinghausen (1891), very often can be seen in the whole length of the septa (Fig. 1). Circulatory disturbances in the interalveolar vessels may also extend through

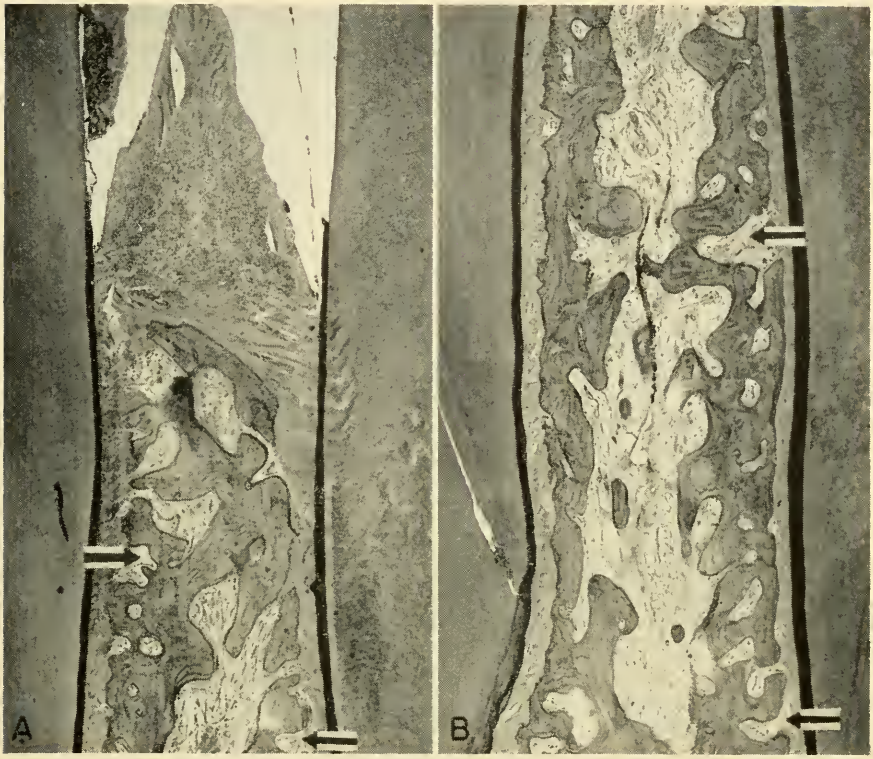

FIG. 1. A, marginal, B, basal part of the same septum. On the left side of the septum a vertical resorption has occurred. Through the whole length of the septum (A and B) there are circulatory disturbances and fibrosis of the bone marrow. Only some pieces of the horizontal bone trabeculae are left. In some places circulatory disturbances and loose connective tissue extend from the bone marrow into the pericementum (arrows). These changes are remote effects of the gingivitis. (× 15.)

their branches into the pericementum of the adjacent teeth, resulting in a breakdown of pericemental fibers (Fig. 1). All these observations support the assumption that alveolar bone resorption is caused by circulatory disturbances.

In two types of periodontal disorder, however, there seems to be no relation between gingival inflammation and loss of alveolar bone. In some patients a chronic gingivitis is not accompanied by break-

down of alveolar bone as shown by x-rays; in others, alveolar bone destruction appears when few or no clinical symptoms of gingivitis are present.

The former type of periodontal disorder is usually seen in growing individuals, seldom in young adults. In histological sections from the periodontia of growing individuals with gingivitis and no loss of alveolar bone, it can be observed that they have alveolar bone resorption, but that a pronounced bone production simultaneously occurs, and often in the same bone marrow space (Figs. 3 and 4).

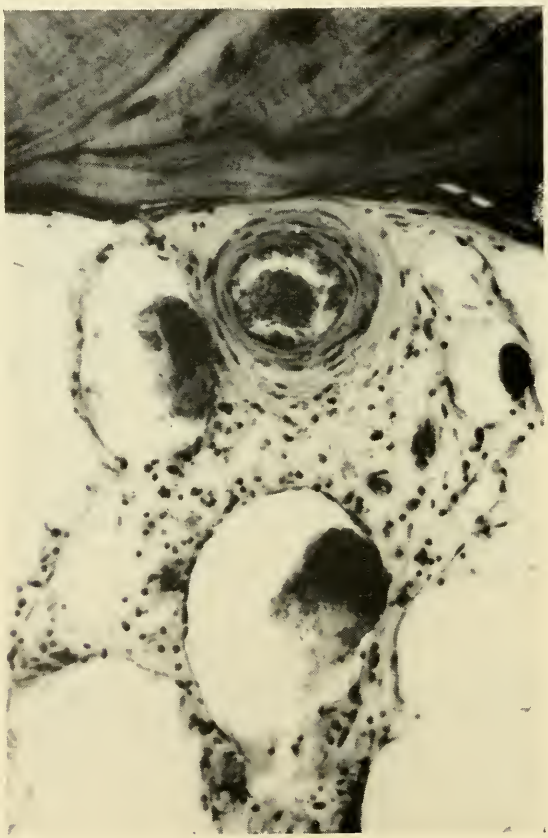

Fig. 2. An extended artery in a septum has compressed a dilated vein. There is a fibrosis between the vessels. (× 200.)

FIG. 3. The marginal part of a periodontium from a 19-year-old individual. Inflammatory changes are seen in the gingiva. The epithelial attachment is located at the enamel-cementum junction. On the top of the septum osteoid has been laid down. In the three marginal round bone marrow spaces bone resorption and apposition are taking place, as seen in Fig. 4. In the other bone marrow spaces there is apposition in spite of the circulatory disturbances and the fibrosis. (× 30.)

Resorption on one side of a bone trabecula and apposition on the other side is also frequently seen.

In these patients the destruction of alveolar bone is compensated or exceeded by alveolar bone formation owing to hereditary factors and to functional impulses. The same bone production is also responsible for the very quick repair of alveolar bone in children after orthodontic treatment.

In young adults with gingivitis and no loss of alveolar bone, the bone formation incited by functional impulses is able to replace the resorbed alveolar bone. This response is seldom seen in adults in our populations. Among east Greenland Eskimos subsisting as hunters far from the white population, it is, however, common to find

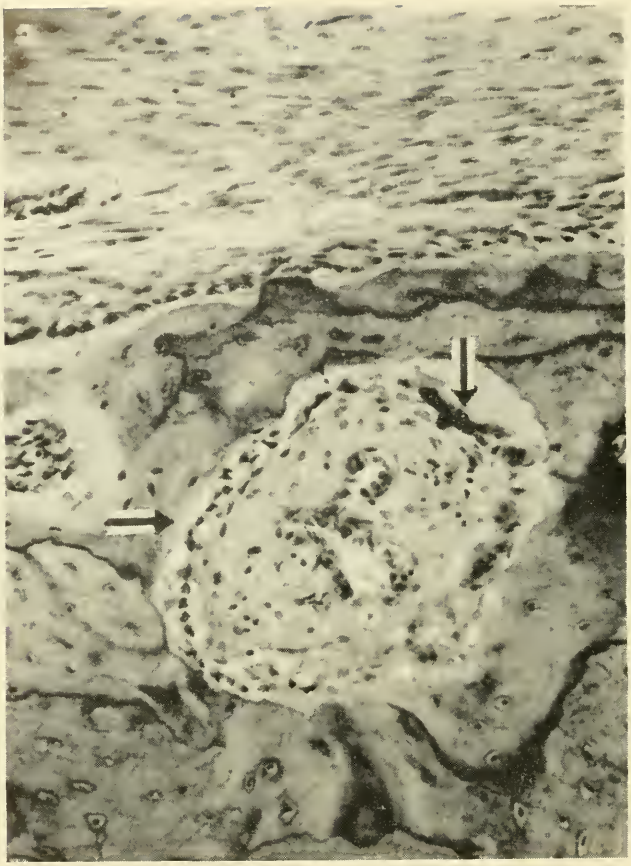

FIG. 4. Osteoclasts (vertical arrow) and osteoblasts (horizontal arrow) in the same bone marrow space as that shown in the marginal part of the septum in Fig. 3. On the top of the septum, osteoid lined by osteoblasts can be seen. (× 150.)

very little or no loss of alveolar bone in adults who have had a chronic gingivitis for a long time. This has been reported by Pedersen (1937, 1939) and Hilming and Pedersen (1940), who also found that gingivitis in the Eskimos on the west coast of Greenland is followed by the same breakdown of alveolar bone that Europeans have in cases of gingivitis. These Eskimos live together with the white population and eat their type of food. The lack of alveolar

bone destruction in the east Greenland Eskimos may be due to the composition and consistency of their food. The latter ensures a very good functional development of the periodontal tissues.

An explanation should also be given as to the loss of alveolar bone when very little or no color change and swelling is present in the gingiva. This type of periodontal disorder can be found in all age groups, more often in females than in males. A clinical examination does not in these cases disclose local factors which can account for the loss of alveolar bone (Seidler *et al.*, 1950). In these patients very often the fixed and the movable gingiva have the same light pink color, with a normal mucous membrane.

In gingival biopsies a regressive inflammation is found, usually with little or no hyperemia and exudative reactions. In autopsy, material bone formation is very rare even in sections from growing individuals.

Patients with anemic gingiva frequently have cold hands and feet and diminished salivation. In some of them an increase in the blood cholesterol up to double the normal amount has been found. It has been suggested by Wang-Norderud (1951) that biopsies should be taken from the palate in the region of the second premolar in cases of anemic gingiva accompanied by advanced destruction of alveolar bone. The biopsies reveal that the lumen of the vessels in the palate usually is reduced owing to thickening of their walls. Loss of elastic fibers and degeneration in the vessels also occur (Fig. 5). Similar changes have been found by Quintarelli (1957) in the vessels of the jaws in patients with periodontal disorders. In biopsies from the palate of diabetic patients the pathological changes in the vessels can be very pronounced.

These histological findings indicate that insufficient bone production is the main cause of the loss of alveolar bone when there is a decrease in the blood circulation in the periodontium.

From the observations mentioned above, the conclusion can be drawn that circulatory disturbances increasing the tissue tonus in the periodontium initiate alveolar bone resorption and that a reduced flow of blood in the periodontium may impede the production of alveolar bone.

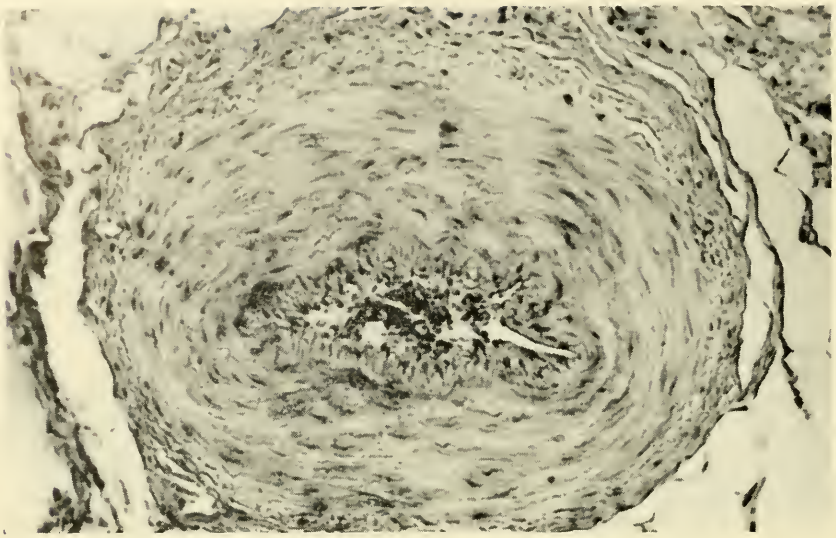

Fig. 5. Reduction in the lumen of an artery, from a biopsy of a patient with anemic gingiva. In the wall of the vessel, which is very thick, atrophic and degenerative changes can be seen. (× 200.)

The Effect of Increased Functional Stress on the Alveolar Bone

It has been explained that the masticatory functional stress increases when alveolar bone is resorbed and that this may incite a compensatory bone formation. A corresponding production of cementum and pericemental fibers also takes place (Reichborn-Kjennerud, 1956).

Mechanical functional stress does not have a formative effect, but a destructive one if it is raised above a certain limit. This has been proved in the previously mentioned experiments by Häupl and Psansky (1938), who by means of activators increased the magnitude and frequency of the intermittent masticatory impulses. When the appliance had been in the mouth for 3 nights, compensatory tissue production was dominant; only in some areas were circulatory disturbances and bone resorption observed (Fig. 6). In sections from patients who had used the activators for 7 nights, extensive

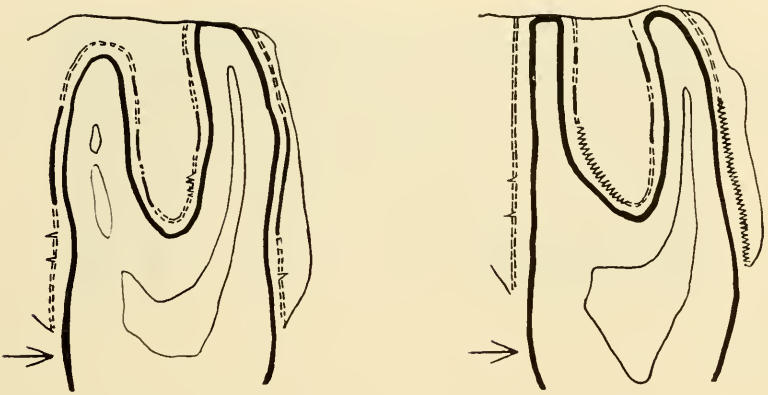

FIG. 6. Drawings of two premolars to demonstrate the reactions after 3 (left) and 7 (right) nights' use of an activator. The masticatory stress has mainly been increased in the direction of the arrows. Where the lamina dura is drawn as a double broken line, apposition has occurred. In the areas where the lamina dura is represented by a solid line, aplasia was found. The zigzag lines indicate resorption areas.

resorption of the lamina dura could be seen in the pressure zones of the pericementum (Häupl and Psansky, 1938; Andresen *et al.*, 1957).

From these and other experiments it is known that circulatory disturbances are the first reaction to an increase in mechanical functional stress above the physiological limit. If the stress is not decreased below this limit in one way or another, the circulatory disturbances are succeeded by atrophy, degeneration, mechanical inflammation, and necrosis (Fig. 7). These alterations which occur in the periodontium and in other organs are due to disturbances in the living conditions of the cells (Häupl and Lang, 1927; Kronfeld, 1931; Coolidge, 1938; Tillotson and Coventry, 1950; Reichborn-Kjennerud, 1956).

That an exposure of the periodontal tissues to constant mechanical stress above the physiological limit will also break down the alveolar bone is seen in orthodontic treatment. In animal experiments where an uncontrolled, often traumatic effect of the masticatory forces is obtained by cementing crowns which are too high on single teeth, alveolar bone resorption also occurs (Gottlieb and Orban, 1931; Wentz *et al.*, 1958). In the same type of experiments in monkeys, Glickman and Smulow (1962) could observe alveolar bone resorp-

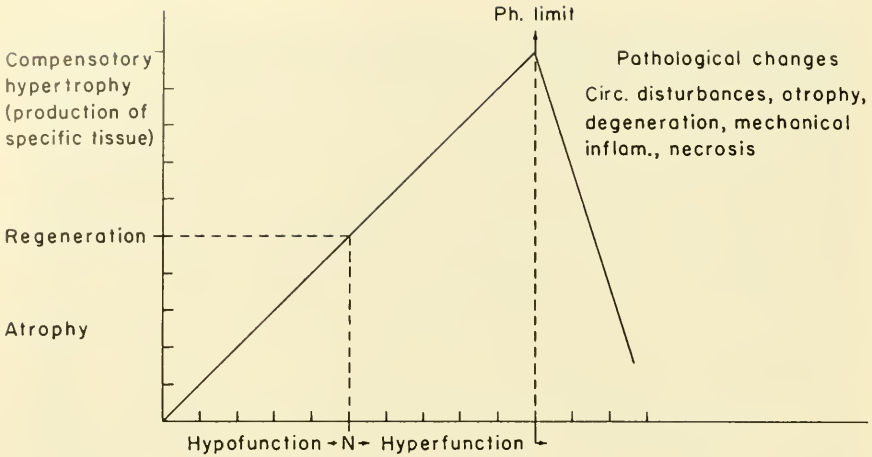

FIG. 7. Abscissa, mechanical functional stress, increasing from left to right. Ordinate, tissue reactions to changes in the functional stress. *N*, normal functional stress activating tissue regeneration. Hyperfunction from *N* to the physiological limit is followed by compensatory tissue formation. Above this limit it results in pathological alterations and destruction of tissue. Production of normal tissues of different kinds decreases rapidly.

tion and development of inflammation in the supra-alveolar gingiva.

It is obvious that an increase of the functional stress above the physiological limit will aggravate pathological changes already present in the periodontium, and it has been found that it influences the spread of a gingival inflammation (Orban, 1954; Macapanpan and Weinmann, 1954; Glickman and Smulow, 1962).

Alterations in the periodontal tissues caused by increased functional stress during mastication can be studied clinically in cases of hyperfunction in the front region due to a bite sinking in the lateral segments (Ramfjord, 1959).

A reduction in the height of the bite in the premolar and molar region may have no effect on the front teeth, which indicates that compensatory periodontal tissue formation has taken place. But very often, and particularly in older persons, the result is increased mobility and migration of the front teeth. These are clinical symptoms of alveolar bone resorption. In the upper jaw, more or less or all of the bone facial to migrated front teeth may be lost (Fig. 8). This destruction is frequently followed by a deepening of the gingival

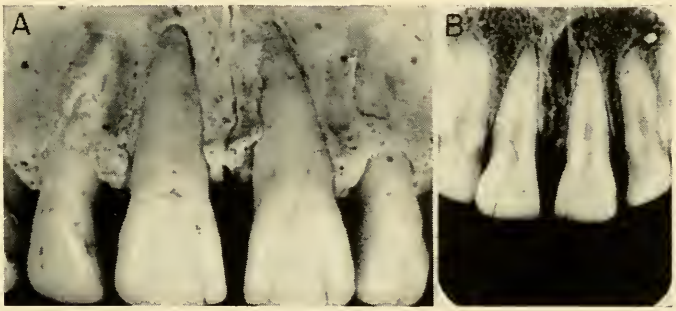

FIG. 8. A, destruction of the maxillary bone in the labial alveolar region due to migration of the teeth following a bite sinking; osteophytes are seen on the facial side of the right lateral incisor. B, x-ray of the teeth in A.

pockets on the lingual side of the migrated teeth. Resorption of the lamina dura of the teeth and of their root tips is also seen.

The progression of these alterations can best be observed in x-rays of the lower front region (Fig. 9). Here the first reaction usually is resorption in the central part of the septa, followed by an extension of their nutritional canals, widening of the apical part of the pericementum, and root tip resorption. When the changes are not accompanied by a breakdown in the marginal part of the alveolar bone,

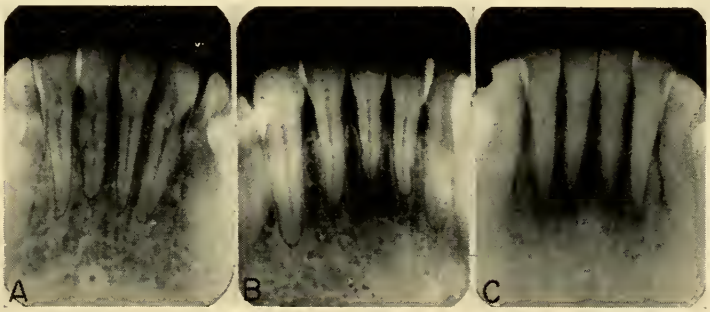

FIG. 9. Reactions to hyperfunction in mandibular incisor region. A, pulp stones in the region of the tooth necks, dentin formation in the crowns. B, pulp stones, dentin formation, breakdown of the central parts of the septa, widening of the apical part of the pericementum, root tip resorption. C, there is very little left of the pulp, the nutritional canals in the septa are visible, the apical part of the pericementum is extended, the root tips are blunt. In A and B there is no marginal destruction of the septa, indicating that the reactions in their profound parts are not due to a gingivitis, but to hyperfunction.

it may be assumed that they are due to increased functional stress.

Another finding sustaining this concept is the nearly constant appearance of pulp stones and the retraction of the crown pulps in the front teeth in these cases. These reactions must result from circulatory disturbances in the apical part of the pericementum due to increased movements of the root tips during mastication.

A clinical symptom which very often precedes and always follows an alveolar bone resorption manifesting itself in an increased tooth mobility is dullness by transversal percussion of the teeth. Histological observations and experiments indicate that this symptom is due to circulatory disturbances in the pericementum (Reichborn-Kjennerud, 1956). Percussion "dullness" is a means of diagnosing an increase in the functional stress above the physiological limit.

It can be concluded that an increase in the masticatory functional stress has the following effects. When it is raised to a certain limit it stimulates a compensatory production of alveolar bone. If it increases above this limit, this reaction does not occur, but pathological changes appear, followed by destruction of alveolar bone. Functional impulses of this type will thus impede the formation of alveolar bone and incite destruction of it.

The Influence of Periodontal and Systemic Conditions on the Loss of Alveolar Bone

Because circulatory disturbances increasing the tissue tonus are followed by bone resorption, all external periodontal conditions contributing to the development of these changes in the periodontium will promote destruction of alveolar bone.

The internal anatomy and the physiology of the periodontium and of all other organs will always influence the initiation and further spread of disorders, and are very often responsible for the relapse of diseases.

The structure of the periodontium may be more or less favorable for the loss of alveolar bone. It is probable that the resistance of east Greenland Eskimos and other peoples to loss of alveolar bone is partly due to the anatomy of their periodontia. Anatomical conditions having the opposite effect are increased permeability and

thickening of the periodontal vessels (Figs. 5 and 10). These and other alterations facilitating the initiation of periodontal circulatory disturbances may be due to systemic disorders.

A very quick change in the physiological periodontal conditions followed by loss of alveolar bone occurs frequently after irradiation treatment of tumors in the jaws (Stafne and Bowing, 1947; Bruce and Stafne, 1950; Shapiro *et al.*, 1960). Gingival inflammations and

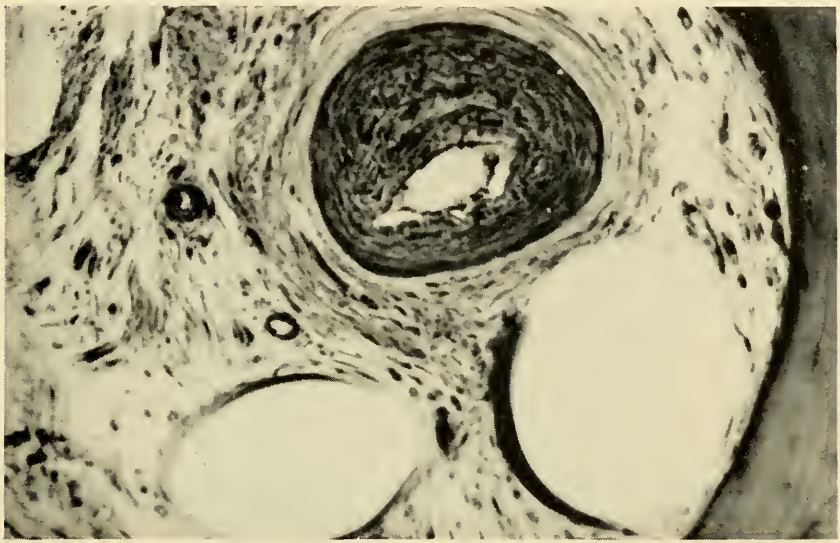

FIG. 10. Circulatory disturbances and fibrosis in a bone marrow space in an alveolar septum of the jaw. A large and a small artery seen in the picture both have a narrow lumen due to thickening of their walls. (× 250.)

advanced destruction of the periodontal bone happen so often in these cases that extraction of the teeth in the region which is to be exposed to irradiation is recommended by many radiologists.

Systemic disorders affect the resorption and formation of alveolar bone in many ways. They can reduce the periodontium's resistance against irritants and thus contribute to the development of gingival inflammations. It is well known that hormonal disturbances, nutritional deficiencies, leukemia, and many other diseases and old age can have this effect (Sheridan, 1959; Brinch, 1937; Boyle *et al.*, 1937; Boyle, 1938; Glickman, 1946, 1948*a*, 1948*b*; Dreizen, 1956;

Duffy and Driscoll, 1958; Burket, 1961; Frandsen and Becks, 1962).

Furthermore, some systemic disorders manifest themselves in periodontal circulatory disturbances, as is the case in scurvy and in hyperparathyroidism. They can also in different ways impede regeneration and repair of alveolar bone and even lead to excess formation of it. Clinical examinations sometimes do not reveal local or systemic conditions responsible for an abnormal alveolar bone picture. It may, however, be due to metabolic disturbances as indicated by observations made by Person (1959). Metabolic studies should therefore be performed in these cases (Barr and Bulger, 1930; Albright and Reifenstein, 1948; Person, 1959; Silverman *et al.*, 1962).

Inherited factors should be mentioned because observations and experiments in animals suggest that they may be of importance for alveolar bone destruction (Baer and Lieberman, 1960; Baer *et al.*, 1961; Shklar *et al.*, 1962).

In summarizing it can be said that periodontal and systemic conditions may influence the loss of alveolar bone by promoting the development and aggravation of periodontal circulatory disturbances, and by preventing formation of alveolar bone.

In individual cases these effects may or may not appear, owing to the duration and the severity of the systemic disorder, and to the influence of many other factors. This explains the controversial reports as to the effect of systemic disorders on the periodontium.

Dental Resorption in Periodontal Disorders

It has been noted that the root tips and the surrounding bone can be resorbed because of circulatory disturbances incited by hyperfunction, which may be the cause of tooth resorptions in other parts of the pericementum.

Tooth resorptions are also seen in the marginal region starting from the gingiva. They appear in various types of periodontal disorders, most frequently in cases of hyperplastic gingivitis. Extensive gingival tooth resorptions are sometimes found in hyperplastic gingival inflammations following a trauma, and in cases of epulis tumors. Gingival resorptions from the connective tissue in gingival

pockets may, however, occur when there is very little or no swelling of the gingiva. This happens frequently on the lingual side in the upper jaw.

In histological sections it can be observed that progressive gingival resorptions usually are caused by fingerlike proliferations of inflamed gingival connective tissue. They may leave trabeculae of dentin in the resorption cavities, giving them a bonelike structure. This happens so frequently that a differential diagnosis between subgingival caries and resorption cavities can be based on this finding. In some cases bone or a hard tissue similar to bone is produced in resorption cavities (Fig. 11).

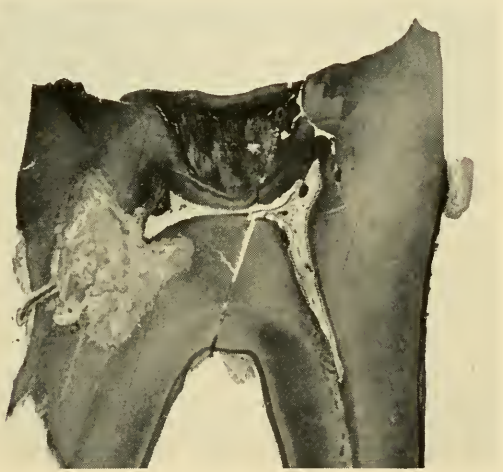

FIG. 11. Resorption extending far into the crown of a molar from proliferating gingival granulation tissue. (× 10.)

Gingival resorptions of the teeth by means of perforating vessels also occur. They may start from the gingival papilla and extend deep into the crown (Figs. 11 and 12). This type of resorption is very often observed in the periodontal bone during the eruption of the permanent teeth.

Resorptions of the teeth in the marginal region relatively seldom appear as compared with the nearly constant alveolar crest resorptions in periodontal disorders. Advanced resorptions of the alveolar

FIG. 12. Detail of Fig. 11. A vessel from the gingiva is entering the resorption cavity, which is filled with connective tissue and bonelike structures. (× 35.)

bone from the pericemental side of the lamina dura may be found while no resorptions or very small ones are seen on the opposing tooth surface.

It has not been explained why the alveolar bone often is resorbed before the teeth, as is also the case in orthodontic treatment. According to histological observations, resorptions do not appear on a root surface where pericemental fibers are intact. This indicates that the fibers must be broken down before the resorption can take place. On the pericemental side of the lamina dura there usually are some places not covered by pericemental fibers, particularly near the canals where branches of the interalveolar vessels enter the pericementum. This may be the reason why alveolar bone is resorbed before the teeth.

In most of the resorption cavities it can be seen that resorption has been followed by apposition of a bonelike hard tissue, which again may be broken down. This is probably due to changes in the tissue tonus in the resorbing connective tissue.

The clinical observations and histological findings mentioned above indicate that tooth resorptions are caused by circulatory disturbances. An explanation has been suggested as to the reason why tooth resorptions occur more seldom than resorptions of the alveolar bone.

Summary

Alveolar bone resorption in periodontal disorders has been discussed in relation to local circulatory disturbances, increased functional stress, and general systemic influences.

1. Histological observations indicate that circulatory disturbances, which increase the tissue tonus in the periodontium, initiate alveolar bone resorption, and that a reduced flow of blood in the periodontium may impede the production of alveolar bone.

2. When the masticatory functional stress is raised to a certain limit it stimulates a compensatory production of alveolar bone, whereas if it is increased above this limit pathological changes appear, including destruction of alveolar bone.

3. Systemic conditions may influence the loss of alveolar bone by inciting and aggravating periodontal circulatory disturbances, and by preventing new formation of alveolar bone.

4. On the pericemental side of the lamina dura there usually are some areas not covered by pericemental fibers, particularly near the canals where branches of the interalveolar vessels enter the pericementum. This may be the reason why alveolar bone is resorbed before the teeth, which, according to histological observations, do not undergo root surface resorption where pericemental fibers are intact.

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12

Bone Remodeling during Dental Eruption and Shedding

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THE process of physiologic resorption and apposition of bone tissue in a *bone* is termed "modeling" or "remodeling" and it is an essential part of osteogenesis. This process begins almost as soon as the primary centers of ossification of a bone are formed and continues throughout life. During the active phase of bone growth, as for example in the tibia of a child, modeling resorption occurs very rapidly, whereas in the adult or later periods of life it slows down. Modeling of bones is species and organ specific, that is, it varies in intensity and location from bone to bone as well as in the same bone of different species. Whereas the process of endochondral as well as intramembranous ossification is identical in different species, the pattern of bone remodeling is markedly different. Thus, at the cartilaginous model stage, the tibia of a dog and that of a monkey may be very similar, but soon after the appearance of the ossification centers and the beginning of remodeling, they quickly begin to assume different shapes. This is also true for different bones in the same skeleton.

In addition to contributing to their morphogenesis, modeling is also important in the functional adaptation of bones. This means that

through modeling and internal reconstruction bones are adapted to the functional stresses placed upon them.

Like the rest of the skeleton, the mammalian jaws are subjected to external and internal remodeling. The process in the jaws, however, is much more dramatic and precise and has more far-reaching effects than in the other bones. This is because the development, eruption, functioning, and shedding of teeth are intimately connected with, and are in part dependent upon, the internal remodeling in the maxilla and the mandible. This interrelationship is the subject of the present discussion.

Bone Remodeling during Dental Eruption

The presence of the epithelial organs, the tooth germs, within the maxilla and the mandible is a unique anatomic feature of these bones (Fig. 1). There are 52 such structures within the human jaws.

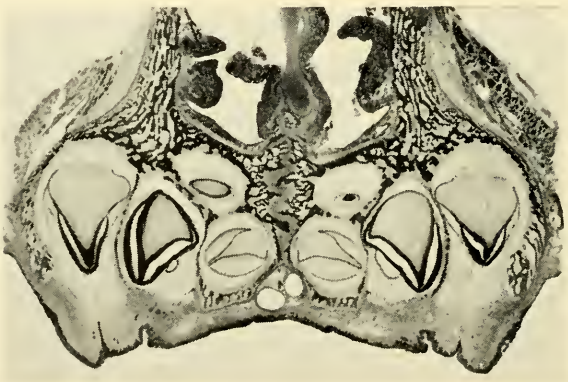


Fig. 1. Frontal section through the maxilla of a fetus 8 months *in utero*. Note eight tooth germs in various stages of development. (× 6.)

The growth and development of each of these organs is somewhat similar to that of the others, but the period of development varies greatly from tooth to tooth. Odontogenesis, or tooth development, begins *in utero* and is not completed for almost two decades. During this long period, tooth germs grow in size, erupt, function, and

adjust their positions within the jaws. In this complicated process, modeling resorption plays a dominant role.

During their life span, the tooth germs and the teeth go through the following major phases (Bhaskar, 1962): growth, movement out of the jaws, and functional adaptation. The remodeling of bone as related to each of these phases will be discussed separately.

Growth Period

The tooth germs originate from the oral epithelium and from there extend into the underlying bone. They are separated from the latter by mesenchyme, and the cavity in which they lie is called the bony crypt (Fig. 2). From this point on, the growth, location, and movement of the tooth germs is dependent upon and closely linked to the modeling changes in the crypt. During the growth

FIG. 2. A maxillary tooth germ with its bony crypt (× 10)

phase, the tooth germ enlarges and the bone tissue of the crypt undergoes rapid bone resorption (Fig. 3). Since the growth of the tooth germs is eccentric and varies from tooth to tooth, the intensity of resorption on the wall of the bony crypt varies markedly in different crypts as well as in different areas of the same crypt. During this phase bone resorption on the crypt wall is striking, but the epithelial tooth germ does not come into contact with bone tissue. It is separated from it by a zone of highly cellular mesenchyme

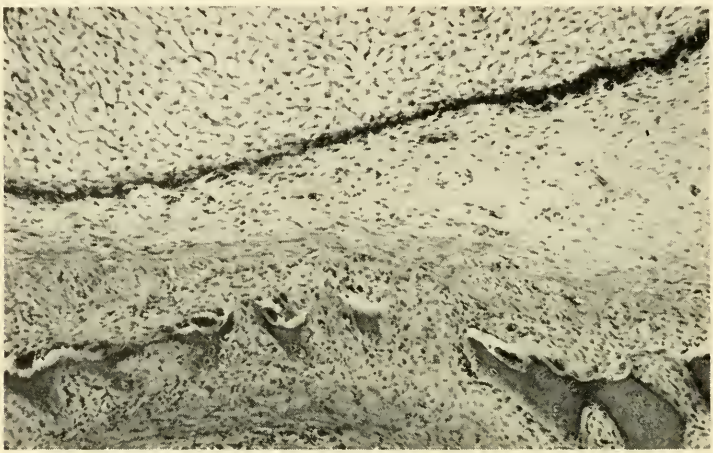


FIG. 3. Osteoclastic resorption of the wall of a bony crypt opposite the epithelial (top) tooth germ. (× 80.)

which in some areas (usually apical) contains a great deal of intercellular fluid (Fig. 4).

Movement Out of the Jaws

After the tooth germ has reached a certain size (completion of the anatomic crown), it begins a pronounced movement toward the oral cavity. The mechanism of this outward migration is not fully known, but the movement is accompanied by rapid bone formation on the wall of the crypt (Fig. 5). As in the earlier resorptive phase, the rate of bone apposition varies in different crypts, in different areas of the same crypt, and at different periods of this phase. During periods of rapid growth the apposition occurs as widely

FIG. 4. A portion of the tooth germ (top) is separated from the bony crypt (bottom) by a wide zone of loose, almost edematous connective tissue. (× 67.)

spaced trabeculae of embryonic bone (Fig. 5), while in areas or periods of slower growth, surface apposition of bone can be seen. The extent as well as the direction of migration of teeth into the oral cavity is intimately related to the apposition pattern of bone in the crypt.

Functional Adaptation

After the tooth erupts and comes into function, the remodeling of the bone of the crypt, now called the socket, continues. It is, however, much less marked. In man, fully erupted teeth undergo wear on their occlusal, anterior (mesial), and posterior (distal) surfaces. This wear is accompanied by an anterior (mesial) migration of all human teeth. The migration is associated with a characteristic remodeling pattern in the socket. It consists of bone apposi-

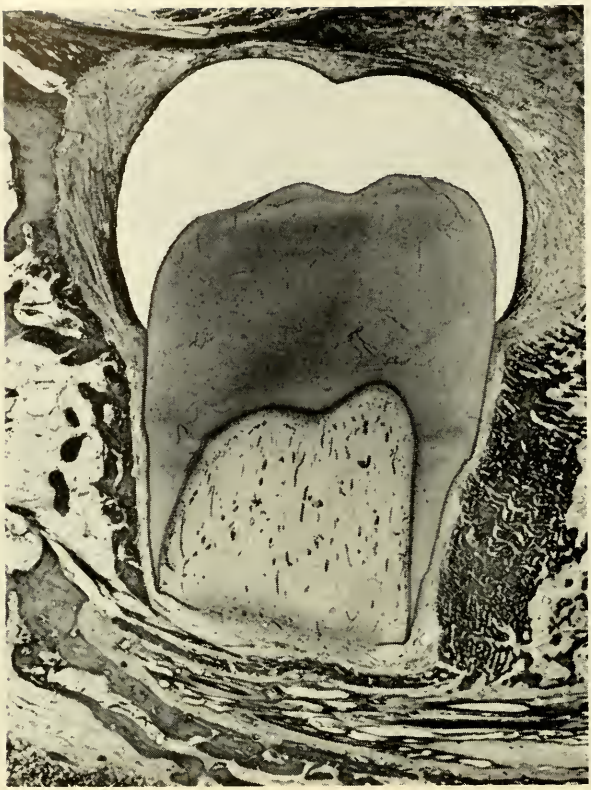


FIG. 5. A molar tooth germ in the mandible. Note rapid bone formation on the wall of the crypt. (× 10.) (From Bhaskar, 1962.)

tion on the posterior (distal) walls and bone resorption on the anterior (mesial) walls of all tooth sockets (Fig. 6A and B). It has been suggested that the anterior migration of human teeth, which is of great importance in maintaining the integrity of the dental arch, is the result of the remodeling of bone in their sockets.

From the brief description above it is apparent that, first, the development and eruption of teeth are intimately associated with the internal remodeling of the maxilla and mandible; and, second, the remodeling process is very intense in the early phases of odontogenesis, slows down progressively, but continues throughout life.

The intimate relationship between bone remodeling and tooth

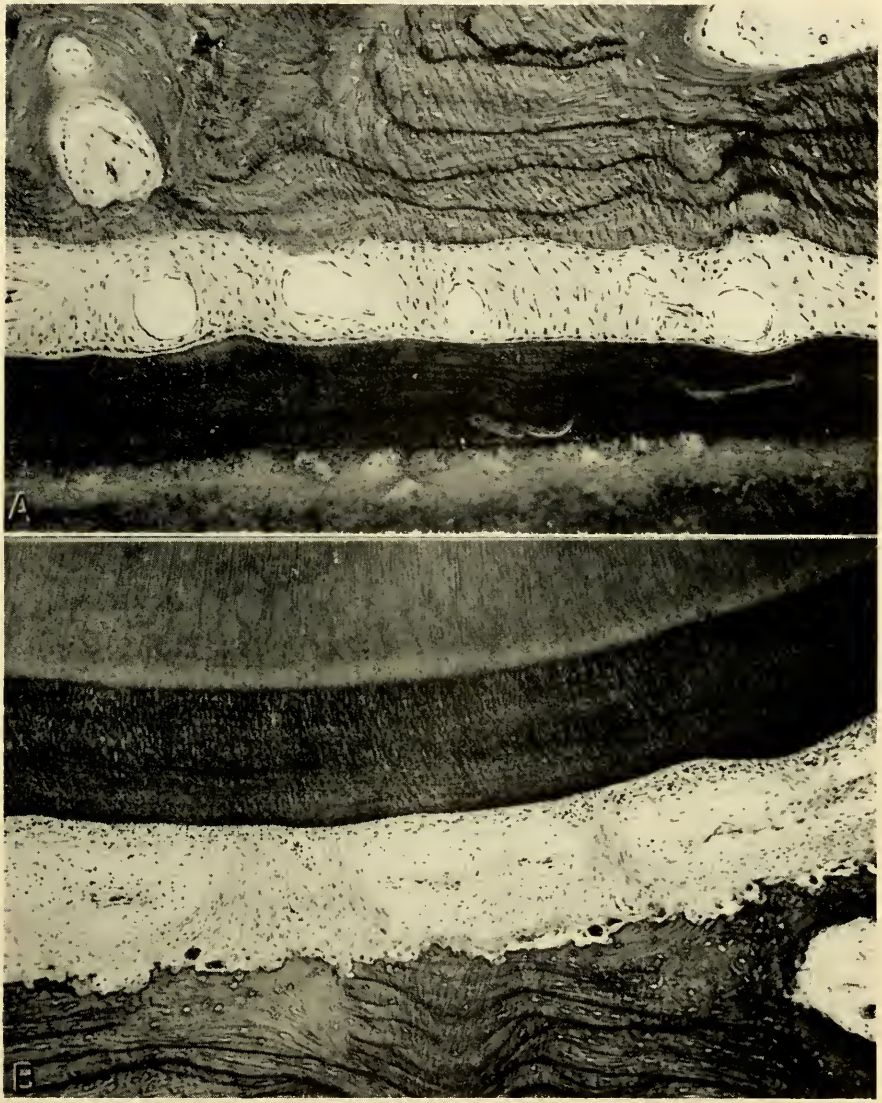


FIG. 6. Upper figure (A) shows the posterior (distal) wall of the socket of an erupted tooth. In this area the bone shows apposition. Lower figure (B) shows the anterior (mesial) wall of the socket of an erupted tooth. Note osteoclasts and bone resorption. (Both figures × 67.)

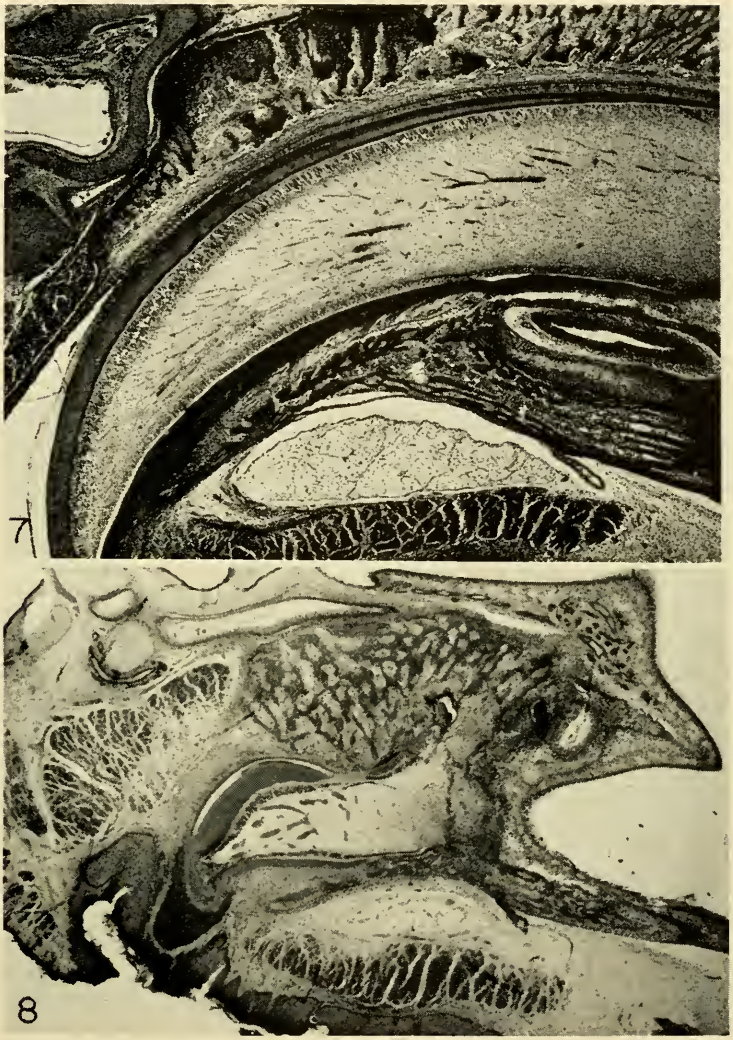
eruption is illustrated by numerous reports. Dramatic illustration of this interdependence is seen in recessive mutations in the rat (Greep, 1941; Schour *et al.*, 1949; Bhaskar *et al.*, 1950, 1952), mouse (Grüneberg, 1935, 1936, 1937, 1938), and rabbit (Pearce and Brown, 1948; Pearce, 1948, 1950*a*, 1950*b*).

Although the changes in these mutations are similar in nature, only those of the *ia* rat will be described.

Dentition and the Bone Tissue of the ia Rat

The *ia* mutation in the albino rat is characterized by a retardation of resorption of bone tissue in the entire skeleton (hereditary hypo-osteoclasis) (Bhaskar *et al.*, 1950; Bhaskar, 1953; Mohammed, 1957). As a consequence, the developing and growing bones show generalized osteosclerosis. In the jaws, the lack of physiologic modeling resorption of the alveolar process leads to the distortion and ankylosis of developing teeth (Figs. 7 and 8) (Schour *et al.*, 1949). The proliferation and differentiation of the odontogenic epithelium is normal. However, because of the failure of remodeling of the bony crypt, the growing epithelial dental organ approaches the wall of the crypt and then extends into the surrounding marrow spaces.

Although the odontogenic epithelium continues to proliferate at a normal rate, the normal growth pattern of the tooth is disturbed owing to the persistence of the adjacent bone trabeculae. The growing odontogenic epithelium follows the course of least resistance and invades the bone marrow spaces in a haphazard and disorderly fashion. The odontogenic epithelium maintains its physiologic potentialities, that is, it differentiates into pulp tissue and odontoblasts, and this leads consequently to the formation and calcification of dentin and enamel. The irregular invasion of the odontogenic epithelium into the bone marrow spaces leads to the presence of strands and islands of dental tissues in different stages of development, on and between the bone trabeculae. This has two consequences. First, the teeth are locked to the surrounding bone, causing ankylosis, which prevents first any movement of the tooth germ and later the eruption of the tooth. Secondly, the continuous growth and the differentiation of the odontogenic epithelium leads to the accumula-



FIGS. 7 AND 8. Incisor and premaxilla of a normal (Fig. 7) and an *ia* (Fig. 8) rat at 10 days of age. The *ia* rat incisor has not erupted. (Both figures × 21.)

tion of the dental tissues and the formation of tumorlike masses.

In summary, it may be stated that the cause of the development of the odontome-like formation in the *ia* rat is the resistance of the bone tissue to resorption in the early days of life. The proliferation

and histodifferentiation of the individual cells of the odontogenic epithelium are not disturbed.

It is apparent that a lack of modeling in the maxilla and mandible not only aborts the eruption of teeth, but leads to formation of odontome-like structures.

The reason for the failure of *ia* bone to undergo resorption is as yet unclear. It has been shown that (1) the *ia* bone does undergo resorption, but this process is slow and delayed (Bhaskar *et al.*, 1950); (2) the *ia* skeleton does have osteoclasts (Bhaskar *et al.*, 1956) (Fig. 9); (3) high dosage of parathyroid hormone promotes

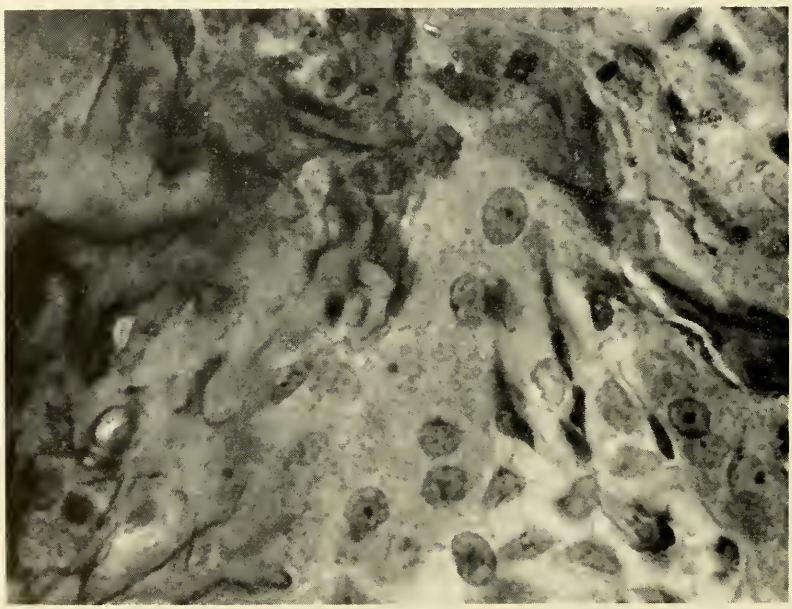
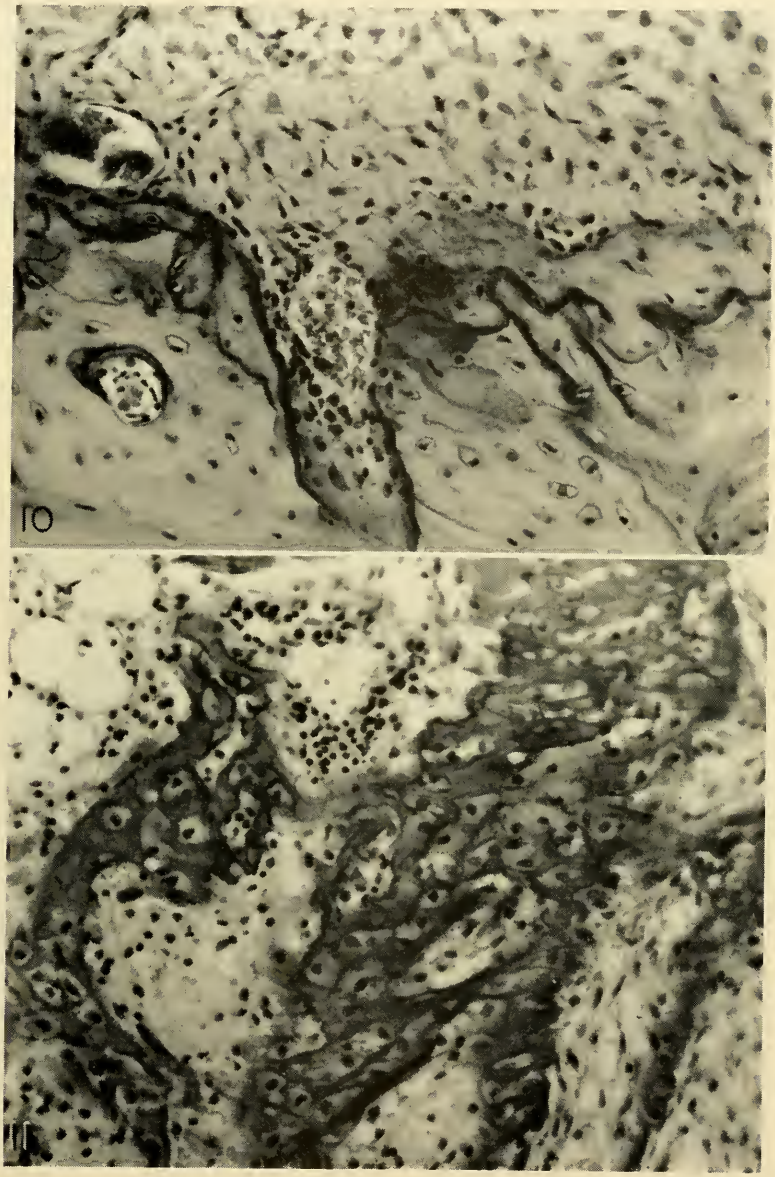


FIG. 9. Bone of *ia* rat with osteoclasts (× 750)

bone resorption and eruption of *ia* teeth (Bhaskar *et al.*, 1952); (4) the *ia* bones are hypercalcified (Kenny *et al.*, 1958); (5) the *ia* defect is not due to hypoparathyroidism (Kenny *et al.*, 1958).

In view of the above findings and in the light of our present knowledge concerning bone resorption, it would appear that hypo-



FIGS. 10 AND 11. Bone tissue of the normal (Fig. 10) and *ia* (Fig. 11) rat at 20 days of age. (Both figures × 300.)

osteoclasis in the *ia* rat is probably due to some defect in the bone tissue itself rather than in the osteoclasts or the parathyroid glands. This belief is strengthened by the fact that grossly the *ia* bone looks more basophilic, is composed of thinner trabeculae, and contains more resting lines than normal (Figs. 10 and 11).

The *ia* jaws demonstrate the complete dependence of normal odontogenesis upon the modeling resorption of the bony crypt.

Bone Remodeling in Shedding of Teeth

Man has two sets of teeth, the primary or deciduous and the permanent or secondary. The deciduous or primary teeth are lost or exfoliated at, or before, the emergence of the permanent teeth. This process is called shedding of teeth. It consists of a progressive re-

Fig. 12. A portion of the human mandible showing the bicuspid teeth (unerupted) and deciduous molars. The bicuspid on the right has already replaced part of the socket of the deciduous tooth. (× 6.)

sorption of first the socket and then the roots of the primary tooth (Figs. 12 and 13), and it begins with the eruption of the permanent successor. As the permanent tooth moves toward the oral cavity, pressure is brought to bear, first upon the alveolar bone tissue which surrounds the deciduous roots and then on the roots of the deciduous teeth (Fig. 14). In the connective tissue intervening between these structures, osteoclasts (or odontoclasts) differentiate and the resorption of the bony socket as well as the root can be seen. One can see such areas as irregular, deep excavations in the root surface

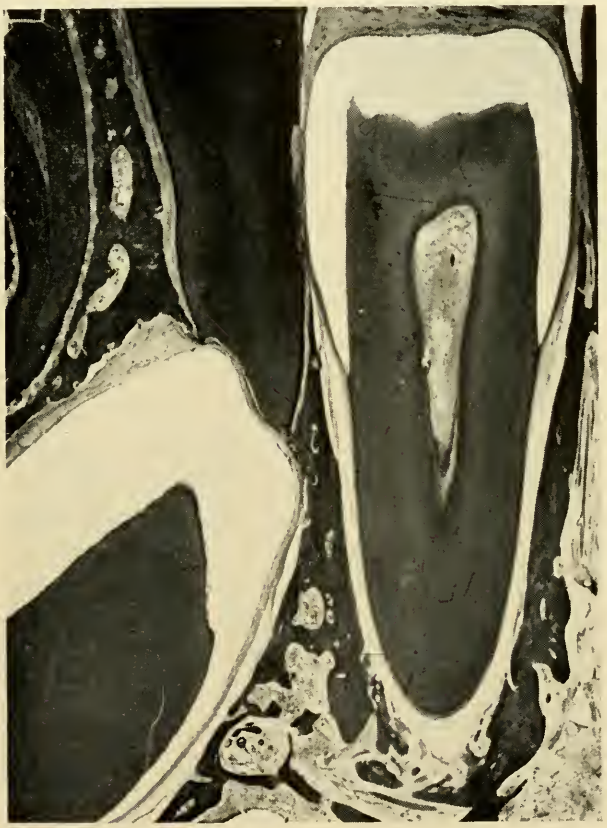


FIG. 13. Not only has the permanent cuspid (left) produced resorption of a part of the socket of the deciduous tooth, but the root of the latter is also undergoing resorption. (× 6.)

FIG. 14. Enamel of the crown of a permanent tooth (lower right) is separated from the root of a deciduous tooth by connective tissue. The deciduous root shows Howship's lacunae. (× 6.)

which contain multinucleated giant cells. It is apparent that the part of the deciduous roots which first undergoes resorption depends upon the location of the permanent tooth and the direction in which it is moving. The process is much more complicated in the area of the bicuspids than it is in the incisor region of the maxilla and the mandible. This is because the anterior teeth are single-rooted, whereas in the bicuspid area the multi-rooted deciduous teeth are replaced by multi-rooted successors.

It is apparent from the description that during shedding of teeth, as in eruption, the remodeling of bone plays an essential role. Not only is the resorption of the bony crypt of the deciduous tooth essential for the eruption of the permanent successor, but after the latter has erupted, the formation of a *new* socket and a new suspensory ligament must occur. Unless this new socket is formed through a process of *remodeling*, the new tooth is virtually useless.

Discussion

During eruption of a tooth a series of very complicated tooth movements occur within the jaws. These are essential so that the tooth may (*a*) keep pace with the growing jaws, (*b*) maintain a certain relationship to other teeth, and (*c*) maintain a certain relationship to the oral epithelium. Although there are only two sets of teeth in man, chronologically, the 26 teeth in each jaw are distinct organs. It is apparent, then, that for a normal dentition, the movement of teeth during and before eruption must be very precise. We have seen that the remodeling of bone is a constant companion to this movement. Whether the remodeling is the *result* or the *cause* of tooth movements is a much debated question. There are two main beliefs. One is that the tooth germ grows and produces pressure on the surrounding bone, which then undergoes osteoclastic resorption, and as a tooth germ moves away from a crypt wall, bone apposition occurs on this surface. According to this concept, bone remodeling within the jaws is a passive phenomenon. The second belief is that the internal remodeling of the jaws is a predetermined genetic pattern, in much the same way as the modeling pattern of a tibia or a femur is a genetically determined pattern. According to this concept, the teeth move as a result of this pattern of bone apposition and resorption. Regardless of which theory is correct, it is undeniable that the internal modeling of the maxilla and the mandible has a great deal to do with the normal development and eruption of teeth.

In the case of shedding of teeth, the eruptive force of a permanent tooth plays an important role in the loss of the deciduous tooth. This eruptive pressure first produces resorption of the bony socket, and then the resorption of the root of the deciduous tooth. These resorptive changes are accompanied by osteoclasts and odontoclasts. The changes which induce the resorptive phenomenon are seen in a multitude of circumstances in the skeleton. Pressure resorption of the bone tissue adjacent to tumors, cysts, aneurysms, and orthodontic appliances are examples of this phenomenon. Regardless of the mechanism, it is certain that bone remodeling is important in shedding of teeth as it is in their eruption.

Summary

It is apparent that during both the eruption and the shedding of human teeth there is an intimate correlation between the changes in the tooth and those in the surrounding bone. During tooth development and growth, the growth of the epithelial dental organ produces changes in the surrounding mesenchyme which lead to resorption of bone. Whether this bone resorption is a result of growth pressure of the tooth germ and surrounding mesenchyme on the bony crypt or is a function of a genetically predetermined pattern of bone remodeling (as in the metaphyseal area of long bones) is difficult to determine. During the movement of teeth out of the jaws, the internal reconstruction of the sockets consists essentially of bone apposition. Whether this process is the result or the cause of eruption has not been established, but its intimacy with the active eruptive phase is unquestioned.

The period of tooth shedding is again marked by extensive remodeling of the alveolar process. The sockets of the deciduous teeth are destroyed by the erupting permanent teeth and rebuilt to adapt to their much larger roots.

In short, the remodeling of alveolar processes of the jaws is a continuous process which begins with the beginning of odontogenesis and does not cease until all the teeth have been lost.

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13

The Deciduous Nature of Deer Antlers

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FEW events in nature rival the unique mechanisms by which the annual renewal of antlers is achieved. The rate at which antlers must elongate in order to attain lengths of up to 4 feet or more in growing seasons of only a few months may exceed 1 centimeter per day, a growth rate probably unequaled elsewhere in the animal kingdom. Once the ultimate dimensions are reached, the entire antler dies, whereupon the velvety skin peels off revealing the compact bone beneath. As nonliving structures penetrating the epidermis (Fig. 9), antlers represent one of the rare instances, together with teeth and the placoid scales of elasmobranchs, in which the continuity of the skin is naturally, though temporarily, interrupted. Serving as weapons and status symbols during the rutting season and for varying periods thereafter, the burnished antlers are shed as the succeeding year's set begin to grow. This is achieved by osteoclastic activity at the junction between the dead bone of the basal part of the antler and the living bone of the frontal pedicle from which the antler originates.

The shedding of antlers represents an extreme instance of a rather common biological phenomenon, usually represented by the loss of certain epidermal structures in various animals following their respective breeding seasons. The pronghorn antelope, for example, sheds the outer keratinized sheaths of its horns annually

(Caton, 1877; Skinner, 1922; Noback, 1932). Among birds a variety of epidermal structures may be shed (Rawles, 1960). The colorful bills of puffins of both sexes are detached from the basal portions after the breeding season, as described by Bent (1919), Forbush (1936), and Lockley (1953). In the male white pelican there is present during the breeding season a cornified crest on the upper beak which, according to Coues (1872), "appears to be shed and renewed in a manner analogous to the casting of deer's horns." These and other more familiar cases of molting involve structures exclusively of epidermal origin. Teeth and antlers, however, represent the sole instances of the natural loss of mineralized vertebrate tissues by shedding. All these cases are examples of how the body gets rid of excess dead tissue from its external surface. The phenomenon of antler shedding, however, has inspired some rather unique interpretations, not the least of which is the notion of Ries (1948) that surplus sexual hormones are stored in antlers during the growing period. Upon the completion of antler growth these hormones are supposedly free to induce rutting behavior. The legendary belief that antlers possess medicinal (including aphrodisiac) properties (cf. Scherbatoff, 1933) is cited as evidence in favor of such an incredible hypothesis. The distinction between truth and myth is not, unhappily, always so obvious.

Mechanism of Antler Shedding

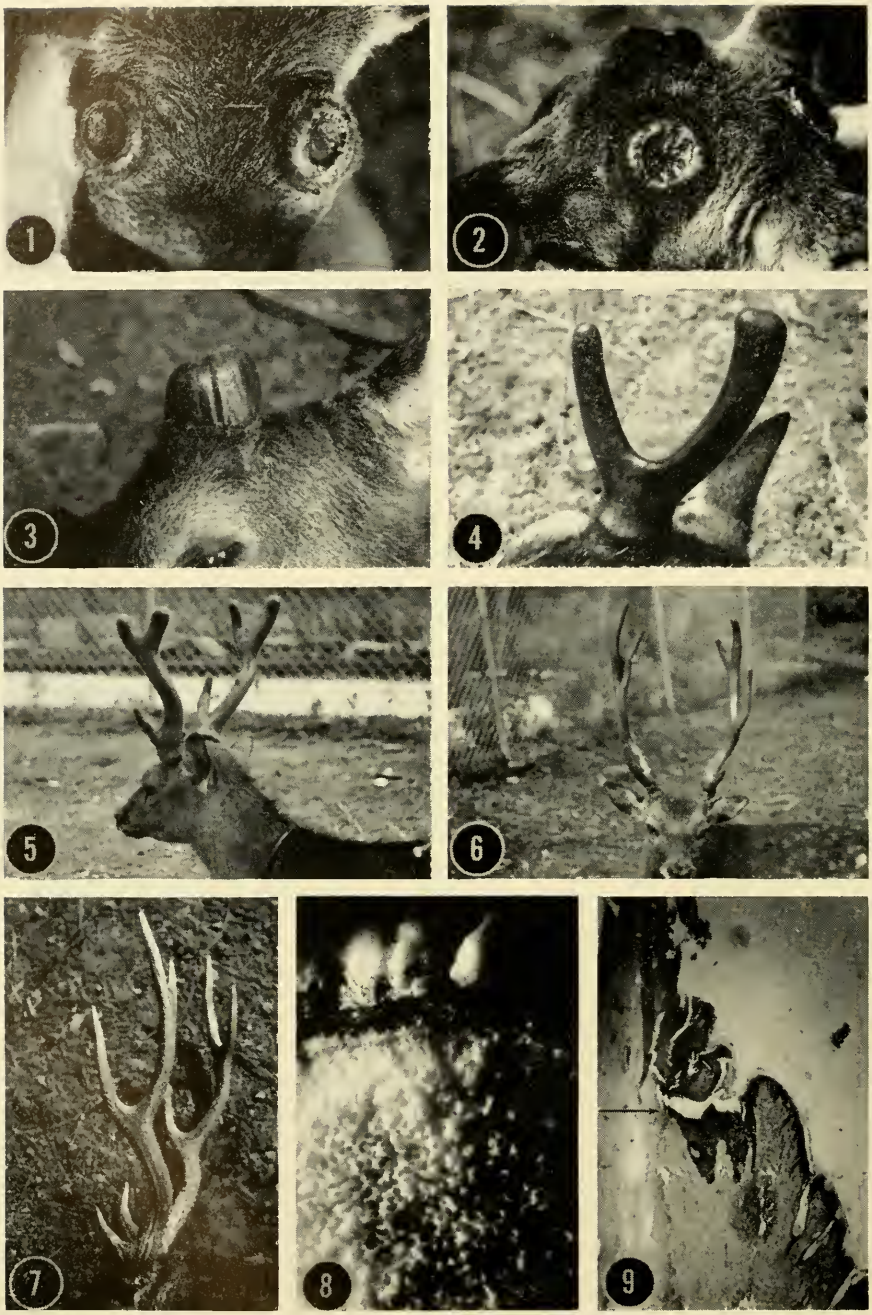
To appreciate fully what is known of the mechanism of antler shedding, one does well to adopt the view of Waldo and Wislocki (1951) that "shedding of the old antler is in reality incident to growth of the new one." In a sense, shedding of the antler represents the crucial point of transition between what Gruber (1952*a*) refers to as the bionegative, or destructive, processes and the biopositive, or constructive, processes in the antler cycle. Indeed, shedding is necessary only because, in the nature of things, death of the antler is the culmination of its growth and development. The normal course of antler development in the sika deer is illustrated in Figs. 1 to 7.

Death of the mature antler is characterized by three principal

events, namely, hardening of the bone, vascular constriction, and shedding of the velvet. These events are set in train by increased testosterone secretion, which also brings about the period of rut following antler death. From this time on there exists, perforce, a union between the compact dead bone of the antler base and the live bony trabeculae of the pedicle. The line of demarcation is at first usually convex, with respect to the pedicle, as is illustrated by the concave base of an antler caused, by castration, to be shed in the early autumn (Fig. 10). During the intervening months the antler apparently dies back gradually, for when shedding occurs in the spring, either naturally or because of castration, its base tends to be convex (Figs. 11 and 12). In either case, the breakage plane is characterized by numerous small bony spicules which are responsible for the rough texture of the antler base (Fig. 8). This base is also remarkable for the normal absence of any traces of blood, a fact indicating that it is the dead, not the living, bone that is eroded prior to shedding.

In 1859–1861, Wyman described the absorption of bone from around the haversian canals just below the "burr," until each cavity united with adjacent ones to effect a separation of the antler from the pedicle. Amplified by the subsequent investigations of Lieber-Kühn (1861), Kölliker (1873), Caton (1875), Macewen (1920), Gruber (1937, 1952*a*, 1952*b*), Wislocki (1942), and Waldo and Wislocki (1951), Wyman's original account still represents an accurate description of the basic process of antler shedding. Kölliker (1873) emphasized that the events preceding shedding involve hyperemia in the frontal pedicle and resorption of haversian lamellae in the central and peripheral regions of the antler-pedicle junction. Thus, in its final days the antler remains attached only by osseous trabeculae in a circular, intermediate zone, a fact that can be verified by close examination of an antler base after shedding (Fig. 8).

In his investigations of roe deer and fallow deer, Gruber (1937, 1952*a*, 1952*b*) described and illustrated the numerous osteoclasts so conspicuously arranged along the trabeculae of the distal end of the pedicle. Under their influence, the bone is eroded and the intertrabecular spaces are correspondingly enlarged to accommodate



FIGURES 1 TO 9

FIG. 10. Sagittal section showing concave base of antler caused, by castration, to be shed on October 25. Note the absence of compact bone at plane of shedding.

FIG. 11. Antler shed on April 2 by a castrated deer, showing convex base.

FIG. 12. Typical convex base of an antler shed in the spring by a normal deer.

the congested blood vessels of the region. Emphasizing the possible importance of neurovascular influences in antler shedding, Gruber (*op. cit.*) says that upon loss of the antler the vessels closest to the shedding zone contract as soon as their contents have flowed out to produce a scab. Beneath this scab, a vascularized syncytial mesenchymal tissue grows out of the marrow cavities of the pedicle. Wound healing and antler regeneration take place subsequently.

Confirming and extending many of the earlier observations, Wislocki (1942) mentioned the existence of a layer of fibrocellular tissue on a Virginia deer pedicle from which the antler had recently been shed. This tissue, probably equivalent to Gruber's syncytium, is located immediately beneath the blood clot and above the cancellous trabeculae of the pedicle. In later studies, Waldo and Wislocki (1951) and Wislocki and Waldo (1953) claimed that this

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FIGS. 1 TO 7. Representative stages in the development of the antler of the sika deer. Figure 1 shows animal's right pedicle shortly after antler was shed; no scab has formed yet, in contrast to opposite pedicle, from which antler was detached a day or two earlier.

FIG. 8. Magnified view of base of shed antler illustrating (top to bottom) the burr, a ring of adherent epidermis, a peripheral zone of relatively smooth bone erosion, and an inner zone of delicate bony spicules. ($\times$ 5.)

FIG. 9. Longitudinal section through distal pedicle and part of antler, showing abrupt termination of pedicle epidermis (arrow) against the antler-pedicle bone (at left). ($\times$ 6.)

fibrocellular connective tissue, with the aid of dermal connective tissue at the periphery, holds the antler to the pedicle for an undetermined length of time between the final dissolution of the osseous antler-pedicle connections and the eventual shedding of the antler. How fibrous connective tissues can hold a heavy antler so rigidly attached to the skull is left to the intuition of the reader. It was further contended that the blood clot forms *before* the antler is shed, a statement which is obviously incompatible with the usually immaculate condition of the antler base after shedding. Having had the good fortune to witness the shedding of several antlers, I can testify to the fact that bleeding does not normally precede shedding. The fresh wound on the pedicle exposes a pink layer of tissue which within a few moments becomes obscured by blood (Fig. 1).

A final misconception in the otherwise laudable investigations of Waldo and Wislocki (1951) is the assumption that shedding eventually occurs, not as a combined result of the weight of the antler and its weakened bony attachments to the pedicle, but because of the upward pressure exerted by the growing pedicle skin on the flangelike burr around the base of the antler. It is true that the distal pedicle skin becomes somewhat tumescent prior to shedding, an indication that incipient regeneration has been initiated, but there is no reason to consider that this plays a mechanical role in detachment of the antler. Examinations by the author of the swollen pedicle skin in sika deer just prior to shedding revealed no indications of pressure being exerted on the burr above. Indeed, circumcision of the distal centimeter of skin from the right pedicles of two sika bucks on March 28 was followed in one case by loss of the operated antler in about 4 days and the contralateral one a few days later. In the other deer, both antlers were shed 3 weeks later, on April 18. It must be concluded, therefore, that the pedicle skin, which is so intimately associated with the histogenesis of the new antler, cannot be directly involved in the shedding mechanism. Inasmuch as the burr is sometimes absent from the very small first antlers of yearling bucks, which are nevertheless shed normally, the role of this structure cannot be to act as a rim against which pressure is exerted. Rather, it has probably evolved as a protective ridge by which the pedicle skin is shielded from injury.

Clearly there is a significant relation between shedding and regrowth of antlers, such that one never occurs without the other. To learn if renewed growth might be affected by preventing shedding, in each of three sika deer the old antler on one side was sawed off within a centimeter of its base, a hole was drilled longitudinally down into the pedicle, and a tantalum screw was tightly inserted (Fig. 13). In two of these deer both antlers were shed within a few days of each other, apparently because the screw was not driven deep enough into the pedicle. In the third deer, however, the control antler was shed in less than a week, but the operated one remained attached for another 7 weeks. Despite this obstruction, the pedicle skin on the operated side developed into antler tissue which

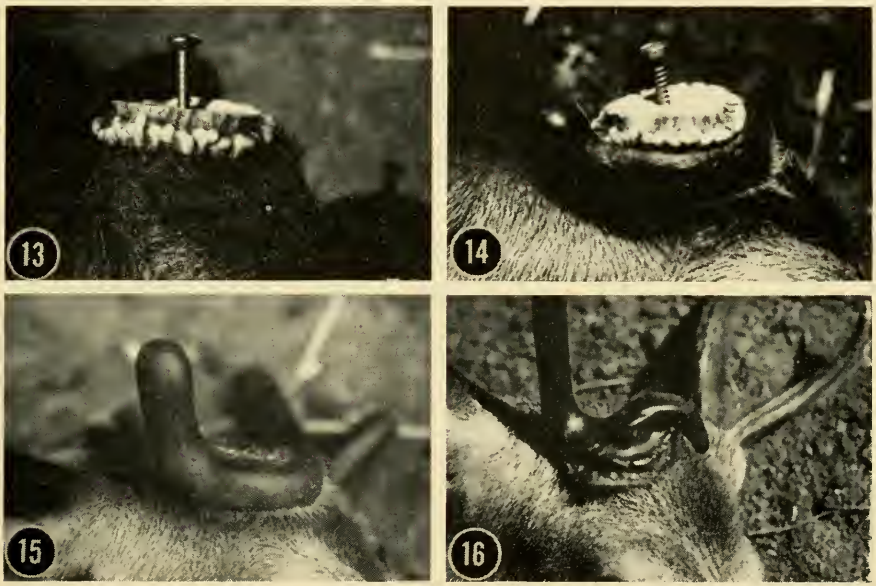

FIG. 13. Appearance of antler soon after it was secured to pedicle with tantalum screw.

FIG. 14. Same antler approximately 1 month later. Incipient antler tissue is bulging around the sides and is beginning to put up a branch from posterior margin.

FIG. 15. Photograph taken on day the old antler was finally lost (7 weeks after opposite control antler was shed).

FIG. 16. At end of summer the posterior branch had elongated considerably, while a curved outgrowth had been produced from the central area.

of necessity bulged laterally on all sides (Fig. 14). The attachments of the old antler to the pedicle were concomitantly disrupted. Nevertheless, this loosened antler remained attached by its screw, while the pedicle skin healed the wound beneath it. Prevented from growing as a direct extension of the pedicle, the new antler regenerated a straight, unbranched protuberance from its posterior margin. When the old antler was finally shed, there was revealed a flattened disc of abortive antler tissue overlying the pedicle (Fig. 15). Apparently in this case growth pressure was responsible for the eventual detachment of the old antler, which in fact had been physiologically shed some 7 weeks before. During the rest of the summer, the posterior branch grew almost as long as the opposite control antler, while another twisted outgrowth was produced belatedly from the center of the pedicle (Fig. 16).

Thus, shedding of the old antler is not prerequisite to regeneration of the new one. Rather, its detachment coincides with the initiation of renewed growth. Indeed, one never occurs without the other, except in cases where replacement of the antler may be secondarily delayed after healing of the pedicle, as in older males of certain species that shed their antlers in the autumn but do not resume growth until spring. Whatever may be the nature of the regulating mechanism(s), it is not improbable that shedding and regrowth are simultaneous reactions to a common stimulus.

Control of Antler Shedding

Influence of Environmental Factors

The seasonal nature of antler shedding strongly implicates the operation of one or more environmental factors as the stimulating agent(s). Mediated through secondary physiological mechanisms, such influences are probably also responsible for many other annual occurrences in deer, e.g., molting, color changes, migrations, and the various events associated with the reproductive cycle. Though each species of deer sheds its antlers at a characteristic season, the variations among different kinds of deer are considerable even in the same geographical area. For example, in the north temperate regions, shedding may occur in the fall, winter, or spring. In adult

sika deer (*Cervus nippon*) and fallow deer (*Dama dama*) shedding most often occurs in May, whereas elk and red deer (*Cervus canadensis* and *C. elaphus*) usually cast their antlers in March. Winter is the time when the moose (*Alces alces*), Virginia deer (*Odocoileus virginianus*), and black-tailed deer (*O. columbianus*) shed their antlers. Still other species lose their antlers in the autumn soon after the rutting season. The European roe deer (*Capreolus capreolus*) sheds in December, as do mature male reindeer and caribou (*Rangifer tarandus*), whereas Père David's deer (*Elaphurus davidianus*) drops its antlers in November.* The pronghorn antelope (*Antilocapra americana*) likewise sheds its horn sheaths in November.

Some species of deer native to neotropical regions also shed their antlers at regular seasons. In India, the Cashmere stag (*Cervus cashmirianus*) generally sheds in March (Blanford, 1888–1891), whereas the hog deer (*Hyelaphus porcinus*) (Blanford, 1888–1891) and barasingha (*C. duvauceli*) (Brander, 1923) drop their antlers in April, though variations from this norm are not uncommon (Mohr, 1932). The Indian muntjak (*Cervulus muntjak*) casts its antlers in May (Lydekker, 1898). Other species may vary according to habitat. Eld's deer (*Cervus eldi*) is said to lose its antlers in June in Manipur and in September in Lower Burma (Blanford, 1888–1891). The sambar (*Cervus unicolor*) generally sheds in March on the Indian peninsula and a month later in the Himalayan region, but individuals may depart markedly from the average (Blanford, 1888–1891).

In all deer there is some divergence between different individuals of the same age and species, and even in the same individual in different years. This variation may become exaggerated among some species of deer native to tropical regions. Thus, in a given locality the male population may represent all stages of antler growth at a given time. The chital (*Cervus axis*) of India, for example, sheds its antlers at any season of the year, as has been noted by Blanford

* According to certain records (Lydekker, 1898; Pocock, 1923; Bedford, 1949, 1952; Wood Jones, 1951) stags have occasionally grown two sets of antlers a year. The summer antlers were shed in the fall, whereupon a smaller set was grown in December, to be shed later in the winter. This phenomenon has not been observed in recent years.

(1888–1891), Lydekker (1898), and Brander (1923). In Ceylon, Phillips (1927–1928) has recorded that these deer also exhibit irregular antler cycles, but that in Southern and Uva provinces 75 per cent of them shed their antlers in April and May. Where the shedding dates are not seasonally synchronized, the other aspects of the antler cycle, as well as the period of rut and birth, are similarly irregular (cf. Zuckerman, 1953). Yet despite the lack of uniformity within a tropical population, there is a strong tendency for individuals to grow successive sets of antlers at approximately 12-month intervals. According to the studies of Valera (1955) on the Philippine deer (*Rusa* sp.), antler shedding by different individuals has been observed at a wide variety of times of year. It is noteworthy that these males nevertheless adhered to annual antler cycles, while the does underwent repeated estrus, sometimes as frequently as every 15 days. In agreement with this is the information generously supplied to the author by Sr. Tomás Blohm (1962–1963) of Caracas, Venezuela, whose careful records of the antler growth cycle in a captive Venezuelan deer indicate that antlers are normally replaced at yearly intervals. In this particular specimen (*Odocoileus gymnotis*), successive sets of antlers were shed on December 8, 1958, December 1, 1959, December 19, 1960, and January 24, 1962. (Velvet was rubbed off in March or April of each year.) The most recent shedding, however, occurred precociously on July 29, 1962, apparently induced by disease (gastroenteritis). Despite this irregularity, the deer did not start to grow new antlers until its usual time of year in December. It then shed the velvet in April 1963, indicating that the basic annual rhythm had not been disturbed.

In temperate species of deer the breeding period is seasonally controlled and coincides in the male with the maturation of the antlers, an occurrence involving the shedding of the velvet, which is induced by the increased secretion of testosterone characteristic of the onset of rut. Should a similar situation prevail among tropical deer, the polyestrous female could mate with only that fraction of the available male population bearing mature antlers. That this is not the case, however, is indicated by Mohr's (1932) account of the hog deer (*Hyelaphus porcinus*) in the Hamburg Zoo: "At first sight

it may seem astonishing that births occur every month, and hence copulation as well, although the bucks have rubbed-off antlers only for a comparatively brief period of the year, and the last of them shed theirs only when the first begin to rub the velvet off in June. As hog deer live in pairs, and the distribution of the sexes is approximately equal, the buck does not have to do much battling to possess its female. Its antlers are not as necessary to it as a weapon as in the larger species, and it willingly and fertilely copulates even when in velvet. The same is true of *Axis* (and *Melanaxis*), where the females are likewise regularly in rut." The validity of this interesting aspect of the problem has been verified by histological examination of testes from 4 Venezuelan deer (*Odocoileus gymnotis*) generously made available to the author by Dr. Pedro Trebbau, Director of the Jardin Zoológico in Caracas. Mature sperm were present in abundance regardless of whether or not the velvet had been shed from the antlers, a condition which contrasts markedly with the seasonal variations in spermatogenesis that were observed by Wislocki (1949) in *Odocoileus virginianus* inhabiting temperate North America.

The data presented by Cabrera and Yepes (1940) indicate that in South America the seasons of shedding are increasingly irregular in proportion to the proximity of habitat to the equator. The huemul, *Hippocamelus bisulcus,* which inhabits the Andes Mountains of Argentina and Chile, sheds its antlers in the winter and grows new ones in the spring. (Gigoux (1929), however, claims that antlers are shed in December (i.e. late spring) in this species.) The pampas deer (*Ozotocerus bezoarticus*), which lives between 5° and 41° south latitude, sheds its antlers at variable times but most frequently in May (i.e. late autumn). The swamp deer (*Blastocerus dichotomus*) inhabiting southern Brazil and adjacent countries does not shed at any fixed season. *Mazama rufa,* the red brocket, inhabits the near-equatorial regions of Brazil, Venezuela, Colombia, and the Guianas. Accordingly, it sheds its antlers at any time of year. In Venezuela, data gathered by the author on *Odocoileus gymnotis* indicate that antler cycles are probably asynchronous among the members of local populations. Comparable information regarding other South American species (e.g., *Hippocamelus antisensis* and

Pudella mephistophelis of the mountains of Ecuador and Peru, and *Pudu pudu* of the Chilean Andes) is unfortunately not available.

At the equator the lengths of day and night are essentially unaltered and equal throughout the year, and the annual fluctuations for some distance on either side of the equator are too insignificant to be of biological importance. Therefore, it is not possible for deer to exhibit annual cycles governed by seasonal variations in day length. Presumably this explains why the members of some tropical species of deer sometimes retain their antlers for periods longer than one year. The earliest account of such an interesting phenomenon was by Forsyth (1889), who wrote (pp. 234–235), "I have taken much pains to assure myself of a fact, of which I am now perfectly convinced, namely, that neither in the case of the sambar nor the spotted deer . . . are the antlers regularly shed every year in the Central Indian forests." Indeed, individual stags were observed not to shed their antlers for successive years in the same locality. With regard to the sambar and the chital, or axis deer, of Ceylon, Phillips (1927–1928) reported that the antlers are renewed annually (though not in unison) for the first few years of life, but thereafter they are shed at variable seasons and may occasionally persist for several years without being replaced. Similarly, Cabrera and Yepes (1940) reported that in the red brocket of tropical South America the antlers may sometimes last more than a year. Finally, Pocock (1912) mentions a South American deer identified as *Mazama bricenii,* which was observed in captivity in England to shed its antlers in April of 1908 and 1909 but not again until May 1911, a period of 25 months having elapsed. Thus, there appears to be sufficient evidence to support the contention that the members of some species of tropical deer may retain their antlers for unusually prolonged periods of time, though they also possess a strong inclination to shed their antlers annually. In the latter cases, however, one wonders how an annual rhythm might be established and maintained in the absence of seasonal diurnal variations. Although it seems doubtful whether animals could recognize and respond to seasonal modifications in the declination of the sun, such meteorological conditions as rainy versus dry seasons suggest themselves as an obvious answer, but experimental proof of this is lacking. Alter-

natively, annual cycles of antler growth and reproduction in the absence of environmental stimuli could represent an atavistic reflection of temperate progenitors. Whatever the explanation, there is reason to believe that when births are distributed throughout the year, the date at which a buck sheds his antlers may bear a regular relation to the time when he happened to be born. Lydekker (1898), noting the persistence of irregular antler and reproductive cycles among chitals (*Cervus axis*) living in England, reported that when birth occurred in December, subsequent antler shedding usually took place each October. Similarly, a deer born in July sheds its antlers in May or June. In the Philippine *Rusa*, Valera (1955) has recorded that fawns begin to grow their first antlers at about one year of age, implying that subsequent shedding may occur a few months before each succeeding birth anniversary. Unhappily, available data on these and other related phenomena among tropical deer are fragmentary and too often dependent on hearsay. Accurate information on the life histories of deer native to equatorial regions is, therefore, urgently needed if the relation of antler growth and shedding to the reproductive cycle is to be more clearly understood. Sufficient reliable evidence along these lines may provide clues as to which endocrinological factors might best be subjected to experimental investigations in attempts to determine how antler loss and regeneration are induced.

In view of the foregoing comparisons between deer inhabiting different latitudes, it would be expected that antler growth cycles (as well as other periodicities, e.g. molting, reproduction) would be responsive to annual variations in day length and that the degree of reaction might be proportional to the amplitude of these seasonal fluctuations. In tropical regions, where diurnal alterations are minimal, deer species tend to be very irregular in the dates at which their antlers are shed. That this is due to an inbred inability to respond to day-length changes, and not to a lack of environmental stimulation, has been demonstrated by the persistence of irregular breeding and antler cycles in such Indian deer as the chital and the sambar after prolonged residence in England (Lydekker, 1898; Pycraft, 1914; Bedford and Marshall, 1942). Unfortunately, I know of no records of the reverse situation, namely, the transfer of temperate

species to tropical zones. It would be interesting to learn if such deer and their offspring would continue on an annual schedule coincident with that of their original habitats in the near absence of seasonal variations in the environment.

Attempts have been made, however, to transfer various species of deer from northern temperate zones to New Zealand (Donne, 1924; Marshall, 1936, 1937). Following such transequatorial shifts of red deer, fallow deer, moose, wapiti, and Virginia deer, the antler and reproductive cycles readjusted to the seasonal reversal. Clearly, these animals shed their antlers in response to the seasonal changes to which they are directly exposed. Indeed, it is possible to change the antler growth cycles according to artificially altered day lengths, as has been proved by Jaczewski (1954). In these classic experiments, red deer which had already begun to grow their antlers were confined in a darkened building from 4 P.M. until 8 A.M. daily. Subjected to suddenly decreased day length from late March or early April until June or July, these animals completed the development of their antlers, shed their velvet, and manifested precocious rutting behavior. Several weeks after they were returned to the normal summertime day lengths, the antlers were shed and new outgrowths were produced during what remained of the summer. The velvet was next shed in September, but antlers were retained until the usual shedding dates the following spring. Thus, it has been possible to induce the formation of two sets of antlers in a single year by the appropriate manipulation of day lengths. Decreases in day length accelerate maturation of the antlers and hasten the onset of rut, whereas increased day lengths stimulate shedding of the antlers in the red deer. Experiments by French *et al.* (1960), however, in which Virginia deer were exposed to artificial 16-hour days and 8-hour nights starting in October, failed to have marked effects on antler growth cycles. Under these conditions, antlers were shed only 2 to 3 weeks earlier than controls despite sustained exposure to prolonged day lengths for $1\frac{1}{2}$ years. The fact that Virginia deer shed their antlers in the winter when the days are short (whereas red deer shed in the spring) may account for the relatively poor response in the former species to increased lengths of daylight. Nevertheless, investigations such as these tend to indicate that

variations in the daily amount of light play a greater role than does the absolute duration of daylight in regulating antler and reproductive periodicities.

Influences of Internal Factors

The control of antler shedding is also affected by certain secondary factors, not the least of which is age. Young bucks bearing their first set of antlers, which are usually unbranched spikes, invariably shed later than do older members of their species. The difference may be rather small, as in sika and fallow deer, in which the yearlings shed their antlers in late May or early June whereas mature animals do so usually in late April or early May. In other species, there is a somewhat greater age difference. Young roe deer usually lose their antlers in January or February, whereas mature ones shed in late November or December. Very old bucks are reported to have shed in late October or early November. In the moose, year-old males drop their antlers in May or June, young adults shed in February or March, fully grown bulls usually lose their antlers in late December or January, and old ones may shed them as early as November. A similar pattern is exhibited among reindeer and caribou, the young males losing their antlers about April and the oldest ones shedding them in late October or early November, with intermediate ages in between. Initiation of new antler growth is deferred until spring in most species of deer regardless of whether the previous antlers were shed in the autumn, winter, or spring. Nevertheless, older animals begin to grow new antlers earlier than younger ones. Thus, the larger the antlers, the longer is the growing season in which they can develop.

In reindeer and caribou, antler shedding is influenced by sex, for females as well as males carry antlers. As mentioned above, adult males shed their antlers in fall or winter. Calves of both sexes, however, do not shed them until spring. In the adult female the antlers are likewise retained until spring, eventually to be shed either just before, or more commonly soon after, parturition. The temporal correlation between these two events is so marked as to suggest a causal relationship. Indeed, it has been noted by Kelsall (1962), Lent (1962), and McEwan (1962) that barren female caribou

usually shed their antlers before fertile ones do, i.e., in the winter or early spring. Therefore, the condition of pregnancy appears to delay the loss of antlers, perhaps because of the heightened production of sex hormones. As will be described below, shedding of antlers in other species of deer can be postponed by injections of either testosterone or estrogen.

There is good reason to believe that the sequence of events leading from day-length changes to antler shedding is mediated by hormones. This is suggested by such correlations as the relation between shedding and rut. In older and presumably more virile males, antlers are shed sooner after the breeding season than in younger animals. In the female reindeer and caribou, one would suspect that antler shedding is stimulated by endocrine factors associated with parturition or lactation. Wislocki *et al.* (1947) suggested that the male counterpart of the lactogenic hormone might logically be involved in antler growth regulation.

The hormonal basis of antler shedding has been most clearly established, however, by investigations of the effects of castration. The profound consequences of castration have been studied by numerous investigators, notably Gaskoin (1856), Caton (1877), Fowler (1894), Rorig (1907), Tandler and Grosz (1913), and Zawadowsky (1926), as well as more recent investigators to be mentioned below. This operation, when performed on fawns prior to the initiation of antler development, precludes antler formation altogether. Its effect on mature antler-bearing animals, however, depends upon the condition of the antlers at the time of the operation. Castration of bucks with growing antlers inhibits shedding of the velvet and results in the permanent retention of viable antlers. But when a deer is castrated after the velvet has been rubbed off and the antlers are burnished and dead, shedding occurs approximately a month later and new antlers are grown which remain in velvet permanently thereafter. Such castrate antlers continue to grow each succeeding spring and summer, adding to what has previously been formed or replacing what may have been lost by necrosis after winter freezing. In this way, rather bizarre headpieces may develop. In some species, such as the sika and fallow deer, the antlers may become crooked outgrowths lacking the normal number of branches. A Virginia deer

which was protected from freezing for a number of years developed numerous accessory branches leading to the production of massive bouquets of antlers (Wislocki *et al.*, 1947). An axis deer which was observed for 5 years following castration (Bullier, 1948) exhibited increasingly abnormal antler morphology as frozen tines were lost and regrown. These and other species of deer tend also to develop unusually thickened bases, from which varying numbers of tuberosities may grow. Excessive thickening of antler branches may lead to the production of "cactus bucks," as described by Mearns (1907) in the mule deer (*Odocoileus hemionus*). Most remarkable of all, however, are the tumorlike masses into which the antlers of castrated roe bucks develop (Olt, 1927; Blauel, 1935, 1936; Bickel, 1936; Kleesiek, 1953). These amorphous outgrowths, known as peruke antlers, may grow down over the animal's head like a wig, obstructing the vision and ultimately resulting in death from necrosis and infections. Such abnormalities may be attributed in part to incomplete ossification in the absence of adequate amounts of testosterone. The pronghorn antelope, incidentally, responds to castration in a comparable manner by failing to shed the horn sheaths. Thus the successive annual increments of horn remain attached *seriatim* in an abnormally curved accumulation of sheaths (Pocock, 1905).

In contrast to the dramatic effects of castration on deer of the usual kinds, reindeer and caribou antlers appear to be considerably less dependent on sex hormones, a condition probably correlated with the bisexual occurrence of antlers in these animals. As in other kinds of deer, castration of males after the velvet has peeled off results in shedding of antlers in 2 to 3 weeks (Hadwen and Palmer, 1922). Males castrated while their antlers are in velvet, however, retain their antlers in this condition until spring, when the skin is lost and the antlers are shed (Tandler, 1910; Tandler and Grosz, 1913; Hadwen and Palmer, 1922; Fisher, 1939). Antlers are grown annually thereafter, although they are heavier, less calcified, and permanently in velvet, and are shed later than normal. Tandler and Grosz (1913) reported that a spayed female reindeer reacted in this same manner. These deer are thus capable of annual renewal of antlers even in the absence of gonads, but how the velvet can be lost in the presumed absence of testosterone remains to be ex-

plained. In other deer, the velvet cannot normally be shed unless testosterone is present, nor are the antlers lost and replaced unless the velvet has been shed.

The premature loss of burnished antlers brought about by castration is unquestionably the result of reduced testosterone. Not only are unilateral castration and cryptorchidism ineffectual (Zawadowsky, 1926; Jaczewski, 1952), but, according to experiments conducted by the author, replacement therapy prevents the shedding of antlers following castration. In these investigations,* 4 deer were castrated in the autumn and injected at operation with 500 mg of testosterone phenylacetate (Perandren)† in microcrystalline aqueous suspension in a concentration of 50 mg per ml. Thereafter for 3 months each deer was injected intramuscularly with 100 mg testosterone twice a week, followed by three final injections at weekly intervals, the last administered 112 days after the beginning of the experiment. Excluding one animal that died accidentally after 84 days of treatment, the remaining 3 deer shed their antlers an average of 151 days after castration, or 39 days after the last injection (Table I). Another 4 deer were similarly castrated, but given 25 mg (250,000 IU) of estrogen (Theelin)‡ in aqueous suspension (5 mg/ml) at operation, and 5 mg of estrogen thereafter according to the same regimen as above. Antler shedding was delayed an average of 141.5 days after castration, or 29.5 days after the terminal injection. In comparison, 14 control animals castrated at various times between September 24 and April 20 shed their antlers an average of 7 weeks later (Table I). Therefore, both testosterone and estrogen can delay loss of antlers in castrated deer, but 3 to 7 weeks after injections of these hormones are stopped, shedding occurs as in castrates not benefiting from replacement therapy. In all cases, new antler regeneration occurred after the old ones were lost, regardless of the time of year. These results extend the earlier

* Experiments were performed on sika bucks (*Cervus nippon*) anesthetized with succinylcholine (Anectine; Burroughs-Wellcome and Co.) in doses of 0.6 to 1.2 mg per deer. Anesthetics and hormones were administered subcutaneously or intramuscularly with automatically injecting projectile syringes shot from a Cap-chur rifle (Palmer Chemical and Equipment Co., Douglasville, Georgia).

† Samples of Perandren generously supplied by Ciba Pharmaceutical, Inc.

‡ Theelin was generously supplied by Parke, Davis and Co.

TABLE I. Effects of Drugs on Antler Shedding in Castrated Deer

Drug	Deer no.	Date castrated	Interval from operation until antler shedding[a] (days)	Interval from last injection until antler shedding[a] (days)
Testosterone	52	11-21	163	51
	53	11-21	149	37
	54	11-21	141.5	29.5
			Average 151	Average 39
Estrogen	57	11-21	132.5	20.5
	58	11-21	148	36
	59	11-21	144.5	32.5
	60	11-21	141	29
			Average 141.5	Average 29.5
Enovid	43	11-21	139	27
	44	11-21	140	28
	45	11-21	139	27
	46	11-21	134	22
			Average 138	Average 26
Norethynodrel	92	10-25	142	30
	93	10-25	157.5	45.5
	94	10-25	154	42
			Average 151	Average 39
Cortisone	99	11-29	61	
Controls	26	9-24	35	
	19	10-7	79	
	28	10-7	49	
	30	10-7	65	
	24	10-29	28	
	51	11-21	37	
	56	11-21	98	
	61[b]	11-21	112+	
	32	11-26	32	
	29	12-31	60	
	41	1-23	21	
	35	2-18	44.5	
	31	3-18	14	
	23	4-20	8	
			Average 48.7	

[a] Figures represent averages of left and right antlers.
[b] Animal died of unknown causes without having shed antlers.

observations of Wislocki *et al.* (1947) and Waldo and Wislocki (1951), who showed that testosterone administered to normal Virginia deer in winter and spring delayed the normal shedding of antlers for several months. They show further that estrogen exerts effects very similar to those of testosterone on the antlers of adult deer, and corroborate the earlier observations of Blauel (1935) that the antlers of castrated roe bucks are not shed until almost 2 months after injections of estrogen (Progynon) cease.

Castration-induced shedding may be simply a withdrawal symptom based upon testosterone depletion. Alternatively, the drop in testosterone levels may be attended by a rise in the amounts of other hormones secreted, one of which might directly stimulate osteoclastic activity at the pedicle-antler junction. Since it is well known that the secretion of gonadotropins from the pituitary increases when the target organs are removed, an experimental attempt to inhibit the secretion of these hormones was undertaken by administering the contraceptive Enovid * to 4 castrated bucks. Injection schedules were the same as in the previously described experiments, starting with 100 mg of Enovid (50 mg/ml sesame oil) at the time of castration and 50 mg per injection thereafter. As shown in Table I, this effectively delayed antler shedding for an average of 138 days, or 26 days after the final injection. However, caution is indicated in concluding that these results represent solely the effects of reduced gonadotropin secretion, for in addition to the active ingredient, 17-ethynyl-17-hydroxy-5(10)-estren-3-one (norethynodrel), these preparations also contained 1.5 per cent of the 3-methyl ether of ethynyl estradiol, which would be expected to mimic the effects of estrogen as described above. To obviate this objection, 3 other castrated deer were similarly injected with purified preparations of norethynodrel * over a period of 112 days. Their antlers were retained an average of 39 days after the last injection (Table I), indicating the possibility that antler shedding may be stimulated by a pituitary hormone, perhaps a gonadotropin.

The hypothesis advanced by Tachezy (1956) that gonadotropin might be responsible for the shedding if not the regrowth of antlers

* The author is especially grateful to G. D. Searle and Co. for their cooperation and generosity in supplying Enovid and purified samples of norethynodrel.

was tested on a final group of 4 deer which were not castrated. These were given injections of 5000 IU of chorionic gonadotropin (Antuitrin-"S"; Parke, Davis and Company) according to the same schedule as before. One animal died accidentally after a month, but the 3 others exhibited no signs of shedding or growth during the course of 4 months and the accumulated administration of 150,000 IU of gonadotropin per deer. Unhappily, such negative results are not necessarily meaningful, for the preparations were highly soluble proteins which might possibly have been effective had they been injected in higher doses at more frequent intervals. Nevertheless, as Wislocki (1943) has pointed out, gonadotropin secretion in normal deer is minimal when shedding and initial antler growth occur. Therefore, its role would appear to be that of stimulating testosterone secretion and spermatogenesis, as the breeding season approaches, rather than influencing antler shedding several months earlier.

The deciduous character of deer antlers cannot be considered in its natural perspective except as a component phase in the annual reproductive periodicity typical of most deer. Hence, the shedding of antlers nearly always occurs sometime after rut and before the season of birth. Thus, newborn animals are not jeopardized by the presence of antler-bearing adults except in the case of reindeer and caribou cows that may not drop their antlers until soon after calving. Another interesting exception to this generalization is represented by the roe deer. Not only does this deer possess mature antlers from early spring (some 2 months before the fawns are to be born) through late fall, but it is unique in that its antlers normally develop during the winter instead of the spring and summer as in other species. Moreover, it mates in July and August but implantation of the ova is delayed until December, approximately 5 months before birth. Considering these various departures from the more common sequence of events, it would be surprising if the roe deer did conform to the reproductive patterns typical for most other deer.

More specifically, the shedding of antlers may be correlated with reduced spermatogenesis and diminished testosterone secretion such as occur after the rutting season (Wislocki, 1949; Stieve, 1950; Frankenberger, 1954). Accordingly, castration brings about pre-

mature loss of antlers in male deer, and parturition is accompanied by shedding in female reindeer and caribou. Common to these two phenomena is an abrupt decrease in the secretion of sex hormones, an effect which might be causally related to antler shedding.

It remains to be determined how the effects of decreased testosterone are related to antler shedding and replacement. Conceivably, simple release from the inhibiting effects of testosterone might bring about shedding. Yet experiments by the author in which 4 normal male sika deer were injected with heroic doses of testosterone propionate (Oreton; Schering Corporation), amounting to 5.5 gm per deer in 6 doses over a 3-week period from September 21 to October 11, failed to induce the shedding of antlers upon abrupt cessation of injections. Thus, a *relative* decrease in testosterone levels from supernormal to normal is not sufficient to mimic the effects of castration on antler shedding.

It is possible that a stimulating effect might be involved in antler shedding. If so, such an influence could be mediated either by nerves or by hormones. With reference to the former, the experiments of Wislocki and Singer (1946) show that denervated antlers grow abnormally (probably owing to injuries resulting from loss of sensory innervation) but that the velvet is lost and the antlers are shed at normal times. Waldo and Wislocki (1951), however, mention certain abnormalities in the shedding mechanism of the denervated antler.

The first step in analyzing the operation of endocrine factors in regulating antler loss and regeneration is to determine what glands might be involved. With the exception of gonadectomies, no endocrine deletion experiments have been performed on adult deer and only a few have been attempted on immature ones. Therefore, one cannot rule out the possibility that almost any gland might secrete an antler-influencing hormone.

Literature on the relation of the thyroid gland to antler growth cycles is somewhat contradictory. Grafflin (1942) noted no significant seasonal changes in thyroid histology in the Virginia deer. Freundová (1955), however, described decreased thyroid activity characterized by low epithelium and large follicles during periods of sexual repose in the red deer. In correlation with the onset of

spermatogenesis the follicles decreased in size, stored less colloid, and exhibited more columnar epithelium. The effects of hypothyroidism have been studied by Wislocki *et al.* (1947), who reported normal antler development subsequent to thyroidectomy of a 2-month-old fawn. Lebedinsky (1939) noted enhanced antler growth in roe bucks which had received thyroxin injections as fawns. In adults of this species, however, Bruhin (1953) was unable to affect antler growth by oral administration of 1 mg thyroxin per day for 1 month during the antler growing period. But in yearling reindeer and fallow deer thyroxin injections induced strikingly greater antler growth than in controls.

Inasmuch as antler shedding is effected by localized erosion of bone, a phenomenon well known to be under the control of the parathyroid gland, it is surprising that the parathyroids of deer have not been more thoroughly investigated. What we do know, however, does not indicate a direct relationship of this gland to antler shedding. Grafflin (1942) reported the lack of histologically detectable annual changes in the parathyroid glands of Virginia deer. Moreover, experiments by the author involving semiweekly injections of 1 gm of powdered ox parathyroid glands into each of 3 adult sika bucks for 8 weeks in the autumn failed to bring about any indications of antler shedding.

There is possible evidence that the adrenal cortical hormones play roles in antler growth. Wislocki (1943) stated that the adrenal glands of male Virginia deer exhibit no histological alterations correlated with seasonal changes. More recently, however, Hucin (1957) has described increased secretory activity in the zona glomerulosa during the period of antler growth in the red deer (a correlation between mineral metabolism and mineralocorticoids), but only slight changes in the activities of the inner zones associated with the rutting season. It is interesting to note that Doutt and Donaldson (1959) have reported the unusual occurrence of a female Virginia deer bearing antlers which might have been attributable to androgens secreted by an adrenal cortical tumor. Cortisone probably does not influence antler development, for Taft *et al.* (1956) reported that ACTH injections exerted little or no effect on the antlers of Virginia deer. The present author has shown that 8 weekly injections of 500 mg of

cortisone acetate into 4 castrated sika deer with antlers in velvet failed to induce shedding of the velvet (in contrast to the effectiveness of testosterone, estradiol, and norethynodrel). Similarly, semiweekly injections of 250 mg of cortisone acetate into a castrated sika buck with mature antlers failed to prevent shedding of the antlers, which were lost 61 days after operation (Table I).

In view of the known effects of testosterone and estrogen on antlers, it would seem especially important to undertake studies of other sex steroids. Waldo and Wislocki (1951) administered progesterone to a normal deer without obtaining noteworthy effects on subsequent antler development. In another animal lacking antlers because of prior castration as a fawn, this hormone failed to bring about antler growth (in contrast to the efficacy of testosterone under similar circumstances). Neither did progesterone stimulate antler growth in a female deer, an effect which has been achieved with testosterone, as reported by Wislocki *et al.* (1947), Aub *et al.* (1949–1950), and Waldo and Wislocki (1951). Progesterone has also been investigated by the present author and found incapable of inducing shedding of the velvet in 3 castrated sika deer given 8 weekly doses of 500 mg.

Partly by default and partly by analogy with other secondary sexual characters, the possible existence of an antler-stimulating hormone of pituitary origin has often been assumed (Wislocki, 1943; Tachezy, 1956). This is supported by the reports of Taft *et al.* (1956) and Hall *et al.* (1960) that hypophysectomy of an 8-month-old Virginia deer resulted in the failure of antlers to grow and in the absence of molting during the next 13 months that the animal survived. Had this deer not yet developed pedicles, it would have been comparable to a castrated fawn, inasmuch as the lack of gonadal development following hypophysectomy would have precluded the development of pedicles owing to insufficient testosterone secretion. Growth of antlers in the adult, however, is not dependent on testosterone, as castration studies have shown. Since this animal possessed distinct, albeit small, pedicles at the time of operation in January (Taft, 1962), its subsequent inability to grow antlers was probably the direct result of hypophysectomy and not of secondary testicular atrophy. Taft *et al.* (1956) also indicated that massive

doses of growth hormone were practically without effect on antler growth, and Blauel (1935) observed that the growing antlers of the roe deer were not influenced by administration of whole pituitary powder. In recent experiments by the author, no effects were observed on the autumn antlers of 2 sika deer injected with 1 gm of lyophilized, defatted beef pituitary twice a week for 2 months.

In view of these incomplete data, the course of research in the future is inescapable. It is to approach the problem of antler shedding in particular, and that of the recurrent annual cycle of antler replacement in general, from a variety of directions. From the comparative point of view, valuable insight into the relationships between antler cycles and reproductive cycles may be gained by seeking fuller knowledge of these phenomena in such aberrant forms as reindeer and caribou, which have antlers in both sexes, and in tropical species of deer, which often exhibit very irregular cycles of reproduction and antler succession. A survey of endocrine influences, by extirpation experiments as well as hormone injections, may reveal how the various phases of the annual antler cycle are regulated. Finally, it will be important to examine more closely the actual mechanism of antler shedding. Since the earliest stages of a process are so often the most important, by learning more about the deciduous nature of antlers our understanding of their genesis and growth may be enriched.

Summary

Antler shedding is accomplished by osteoclastic erosion of the haversian lamellae along the line of demarcation between the dead bone of the antler and the living bone of the pedicle. It is an integral part of the sequence of histological events associated with the initiation of renewed antler growth.

In temperate regions, male deer lose their antlers in a fixed season depending upon the species. This is determined by reactions to seasonal changes in day length, as has been proved by noting responses to transequatorial shifts or exposure to experimentally altered day-to-night ratios. Tropical deer show a tendency toward asynchronous, and occasionally prolonged, antler replacement periodicities, to-

gether with a loss in correlation between antler and reproduction cycles.

Antlers are shed earlier in older deer than in younger ones. Delayed by pregnancy, shedding coincides with the calving season in female caribou and reindeer. Castration of deer with mature, dead antlers induces precocious shedding and subsequent regrowth of antlers that remain permanently in velvet. Castration-induced antler loss is precluded by injections of testosterone, estrogen, Enovid, or norethynodrel, but not by cortisone. In normal deer, antler shedding could not be induced by repeated injections of chorionic gonadotropin or of pituitary or parathyroid preparations, nor by abrupt cessation of testosterone injections. Hypophysectomy, however, appears to prevent antler growth.

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14

Internal Remodeling of Compact Bone

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BONES increase in length by the functioning of the growth apparatus, including the epiphyseal cartilage plate and the metaphysis of the long bones. They increase in diameter by apposition of new periosteal bone, while the marrow cavity is being enlarged by resorption at the endosteal surface. In addition to the changes in size and shape of the bones, as a result of the remodeling incident to growth, there is continuous internal remodeling throughout the life of the individual; this serves an important physiologic function, essential to homeostatic control of the calcium level in the blood plasma, and therefore to life itself.

In compact bone there is first the formation of absorption cavities, described by Tomes and De Morgan in 1853. The appearance of these cavities is associated with the presence of osteoclasts, and the cavities are extended until they assume the form of tunnels. Because of certain similarities to rock boring by biologic organisms, as described elsewhere in this volume (chapters 1, 2, and 3), and because of the possible significance of absorption or resorption cavities in the evolution of the skeleton, it seems desirable to treat the sub-

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ject of internal remodeling of compact bone as a topic in the comparative biology of calcified tissues.

Neither rock boring nor the excavation of tunnels in compact bone should be regarded solely as a destructive process; both serve to perform physiologic functions, and can only be understood by consideration of the cycles in which they participate. The rock borers and the boring gastropods engage in the removal of calcified tissues in search of either shelter or food, or both. The tunneling in compact bone, by osteoclastic resorption, is unique in that it is followed by reconstruction of new osteons, or haversian systems, which provide a continuing supply of new and reactive bone, meeting the metabolic needs of the organism while leading to maturation and formation of structural bone.

The internal structure of compact bone has been well described for more than a century; its haversian systems and canals, providing for blood vessels, and its lacunae, housing the osteocytes, with interconnecting canalicules, are easily demonstrable in ordinary histologic sections. Such sections, however, do not reveal any contrast in the density of the numerous osteons and interstitial lamellae that make up the mass of bone. It has also been known that there is an exchange of ions between the bones and the circulating fluids of the body, but not until Chievitz and Hevesy, in 1935, exploited radioactive phosphorus, P^{32}, in the study of bone was the rapidity of this exchange recognized, and its physiologic significance is still under study, largely by means of tracer elements.

With the advent of microradiography, and especially when it was employed together with autoradiography, it was found that the bone mineral, instead of being distributed homogeneously in compact bone, exhibits wide variation in the density of the osteons. Figure 1 shows a low-power microradiograph of a transverse section through the shaft of the tibia of adult man, in which every gradation from beginning calcification of new osteons to maximum density of mature and fully mineralized osteons appears. Figure 2 illustrates two osteons from the radius of a dog, the same section being viewed at high magnification by four different methods: A, unstained histologic section; B, microradiograph; C, autoradiograph, recording alpha tracks from radium-226, administered 2 days before sacrifice;

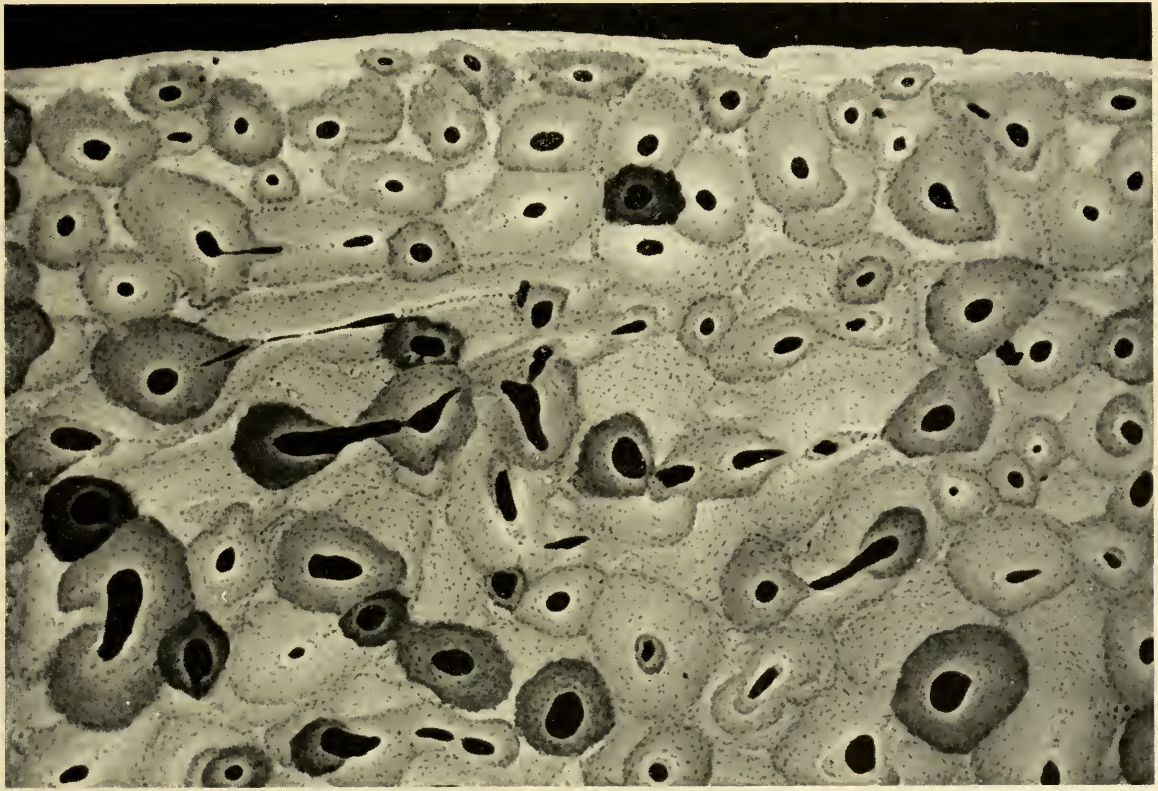

FIG. 1. Microradiograph of a portion of a cross section of tibia from adult man, age and sex unknown. (× 50.)

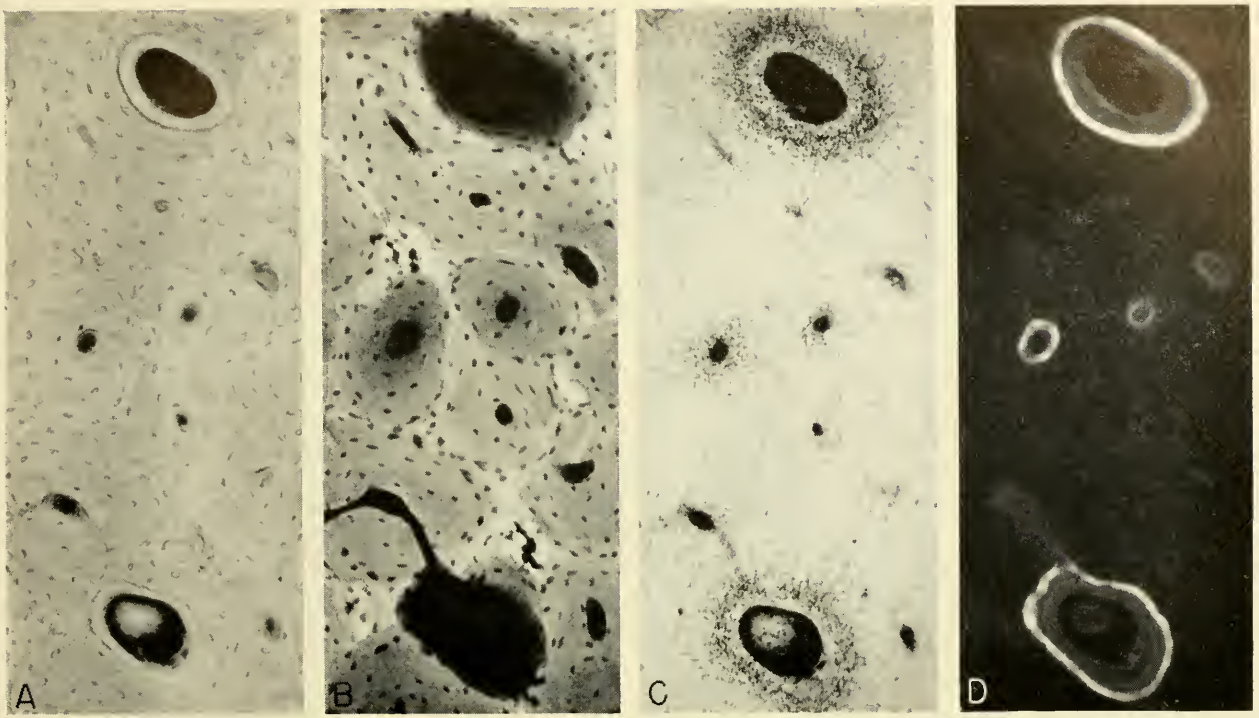

FIG. 2. Four illustrations of the same section of compact bone from the radius of a dog. The bone is from an 8-month-old beagle, given tetracycline, 25 mg/kg, 4 days, and Ra^{225}, 40 μc, 2 days before sacrifice, both intravenously. The bone was embedded in methyl methacrylate and the section, 100 microns thick, was cut with a high-speed rotary saw, without further treatment. A, photomicrograph of unstained section. B, microradiograph, made with 9-kv x-rays on Eastman Kodak 649-O spectroscopic plate. C, alpha-track autoradiograph, made with stripping film, Kodak Limited AR-10, exposed for 14 days. D, fluorescent image with ultraviolet light, photographed on Kodak Panatomic X film. (All images × 140.)

and D, fluorescent image with ultraviolet light, revealing deposition of tetracycline administered 4 days before sacrifice. Treatment of sections of undecalcified bone by these different methods adds greatly to the information to be obtained from their study. The autoradiographs and the fluorescent image reveal that both radium and tetracycline are preferentially deposited in preosseous tissue, as bone is laid down in concentric layers in the rebuilding of osteons.

When visualized in three dimensions, the tunnel, or resorption cavity, is seen to contain blood vessels and connective tissue cells. It is excavated in the long axis of the bone, cutting through old osteons and lamellar bone, without relation to the previous structure. While resorption is in progress, the cavity is lined with osteoclasts; when rebuilding begins, these are replaced by osteoblasts. The tunnel is then filled in, from the walls toward the center, by apposition of bone in successive layers or lamellae, incorporating the osteocytes, their lacunae, and the canalicules. The formation of new layers continues until the canal reaches its final diameter, usually approximately 20 microns; at this time the osteoblasts assume a resting form, and are no longer recognizable.

Using lead as a marker, Vincent (1957) has determined the rate of formation and of maturation of new osteons. An average resorption cavity in a dog takes roughly 3 weeks to form, this being the period required for tunneling or excavation. The building of the new osteon, including partial mineralization of the organic matrix, requires some 6 to 12 weeks. Primary mineralization, to about 70 per cent of the final density, occurs rapidly during and immediately after the deposition of new layers of organic matrix; completion of secondary mineralization, to maximum density, takes much longer and has been found to be incomplete for as long as 18 weeks.

Frost *et al.* (1960), using tetracycline as a marker, found the mean osteon formation time in a 57-year-old man to be 5 weeks. The biologic half life of the osteons was calculated as 2.7 years for the femur and 8.6 years for the tibia. In another study, also with tetracycline, Frost and Villanueva (1960) report that an average of 0.9 micron of new osseous tissue per day is formed in actively forming haversian systems in man.

As to the formation of resorption cavities, little is known of the

mechanism, except that it appears to be identical with osteoclastic resorption in the removal of bone throughout the skeleton. Here the situation seems to be analogous to that in rock boring; in both instances there is speculation concerning the chemical factors in dissolution of calcified tissues; in both cases acid formation and chelation are given consideration as possible mechanisms; in neither have the factors participating in destruction of the hard tissue been elucidated. Rock boring by biologic organisms and tunnel formation by osteoclastic resorption are sufficiently similar in their nature and their manifestations to suggest a common pathway in the evolution of hard tissues.

In connection with the comparative biology of calcified tissues, Amprino and Godina (1953) reported that remodeling of compact bone in the teleost *Thynnus thynnus* L. is initiated by the formation of tunnels by osteoclastic resorption. On the other hand, Norris *et al.* (1963), studying the sea bass *Epinephelus striatus* (Bloch), observed resorption apparently in the absence of osteoclasts; primary bone was penetrated by blood vessels, forming tunnels. Acellular bone, arranged circumferentially around thin-walled blood vessels, was then laid down by osteoblastic activity, a thin sheet of osteoid appearing beneath a single layer of osteoblasts. The diameter of the lumen of the acellular osteons varied from 28 to 41 microns.

There remains the important problem of physiologic regulation. At least two regulatory processes are involved: (1) regulation of initiation and formation of resorption cavities or tunnels; and (2) regulation of the transfer of calcium in such a manner as to maintain the constancy of the Ca^{++} concentration in the blood, in spite of the rapid turnover of the plasma calcium.

Little is known concerning the regulatory control of the formation of resorption cavities. Jowsey *et al.* (1958) have studied the remodeling of compact bone in parathyroidectomized dogs, and have found that tunnel formation continues after removal of the parathyroid glands, although at a somewhat reduced rate; in any event a continuing supply of reactive bone is maintained, adequate to support life, and osteoclastic resorption continues. Since osteoclastic resorption, incident to the growth of bone, also continues in the absence of the parathyroids, it appears that osteoclastic activity is not

wholly dependent upon parathyroid function; a rate-determining relation between the parathyroids and osteoclastic activity seems likely. It is not known whether in the absence of the parathyroids vitamin D assists in promoting the resorption incident to remodeling; such an influence is a possibility.

Once a tunnel is completed, there is a reversal in activity, and osteoblastic deposition of bone begins; there is established a continuing supply of preosseous tissue, and deposition of bone mineral in the organic matrix is initiated. It is in these locations that the reactivity of the newly formed tissue is demonstrable by observation of the uptake of radioactive calcium or radium. When new tissue is laid down, gradually filling in the cavity until only enough space is left for the haversian canal, there is always a fresh surface lining the forming haversian systems, available for deposition of mineral. It is in such locations that accretion continues throughout life.

Deposition of new mineral, as seen by autoradiographs following administration of Ca^{45} or Ra^{226}, is concentrated in these newly formed layers of preosseous tissue, lining the haversian systems; these areas have acquired the designation *hotspots,* as illustrated in Fig. 3. The mineral so deposited is often referred to as exchangeable, labile, or reactive; there is as yet no direct evidence that it is from these locations that mineral leaves the bone to replace that lost in the rapid turnover of the blood calcium. In addition to the concentration in hotspots, there is diffuse deposition of the isotopes, in much lower concentration and more or less uniformly, throughout the compact bone; this has been designated as the *diffuse component* (Marshall *et al.,* 1959). Of the total amount deposited in the skeleton, following administration of a single dose of Ca^{45} or Ra^{226}, approximately one-half is distributed in the diffuse component, while the other half is concentrated in the hotspots, occupying only a small proportion of the total space in the bones.

Bauer *et al.* (1955) accounted for the transfer of calcium between blood and bone, in both directions, by a combination of *accretion, resorption,* and *exchange.* The mineral concentrated in the newly forming osteons, as demonstrable by the use of tracer Ca^{45} or Ra^{226}, is attributed to accretion. Resorption, associated with the presence of osteoclasts, is generally believed to result from an osteolytic action

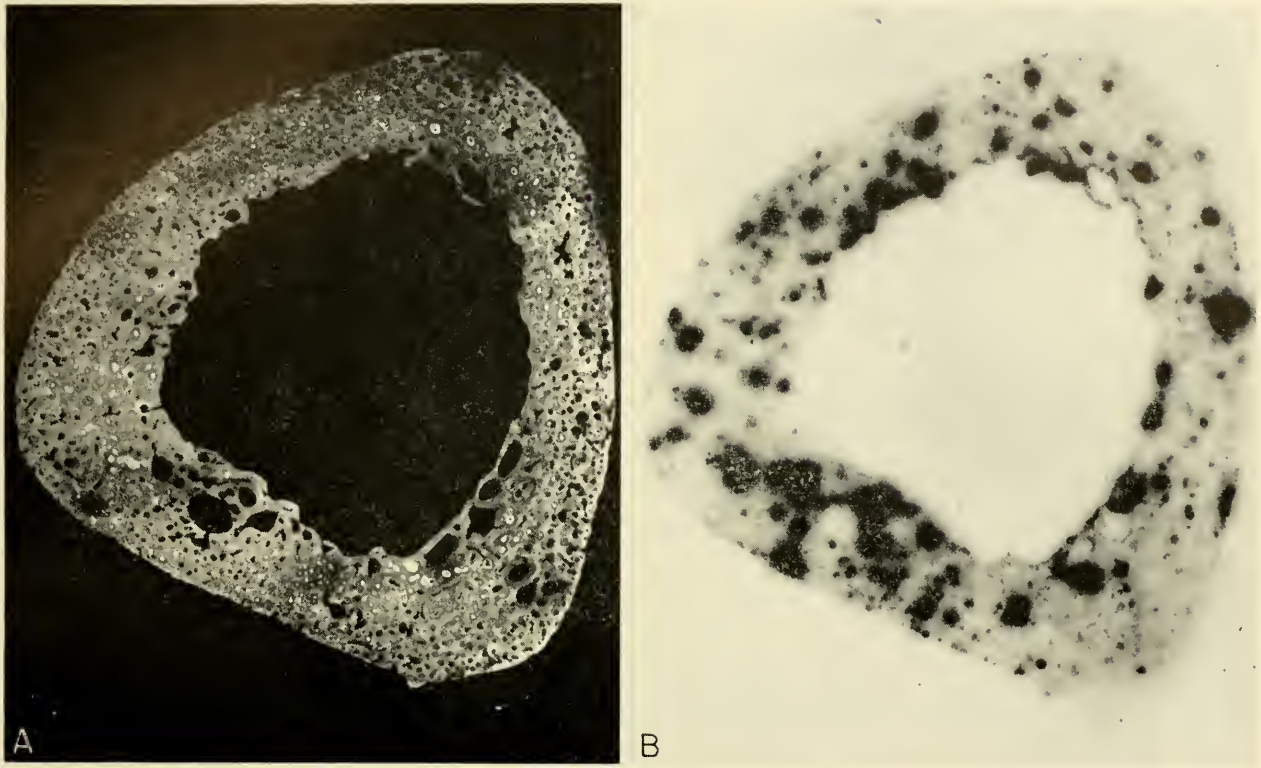

FIG. 3. A microradiograph (A) and the corresponding autoradiograph (B) of a cross section of tibia from a patient having a body burden of 10.5 μc Ra^{226}. The radium was administered orally for medical treatment. The autoradiograph was exposed for 1 year on Kodak type A plate. ($\times$ 6.)

of these cells upon both the mineral and the matrix of bone; it occurs mainly on the surfaces of bone and in the formation of resorption cavities; there is also some indication that osteocytes may contribute to the mobilization of bone mineral. The diffuse component, visualized by administration of tracer elements, is regarded by Marshall *et al.* (1959) as representing the portion of the mineral undergoing *long-term exchange.*

It has been stated above that there is a rapid turnover of the calcium in the blood; this is so rapid that in adult man one out of four calcium ions leaves the blood every minute (Bauer *et al.*, 1961). The calcium can go only into the skeleton, and is replaced by other ions from the bones. For the minute-to-minute equilibrium between blood and bone, it is believed that this transfer occurs by ion exchange; neither osteoclastic resorption nor long-term exchange is sufficiently rapid to account for it. The source of the calcium transferred from bone to blood, by ion exchange, has not been definitely localized, although the opinion is held that it comes from the recently deposited mineral in the osteons undergoing mineralization. These osteons constitute the reactive or *metabolic bone;* in contrast, the fully mineralized bone, in which deposition of new mineral occurs only as the diffuse component, is designated as *structural bone* (Vincent and Haumont, 1960). There is a pronounced difference between the amounts of stable and of labile, reactive, or exchangeable bone mineral; less than 1 per cent of the mineral is described as exchangeable (Neuman and Neuman, 1958).

McLean and Urist (1961) have proposed a dual mechanism to account for homeostatic control of the calcium ion concentration in the body fluids, including the blood plasma. The function of the parathyroid glands is to monitor this concentration. This function is illustrated, in cybernetic terms, in Fig. 4. The parathyroid glands respond to changes in the Ca^{++} concentration in the plasma by altering the output of parathyroid hormone; the net result is that this concentration is held, in normal man, at approximately 10 mg per 100 cc. It seems certain that the effect of the parathyroid hormone on release of calcium from the bones is mediated through the cells of bone, although the mechanisms by which this is accomplished are not fully understood. The three cell types—osteoblasts, osteo-

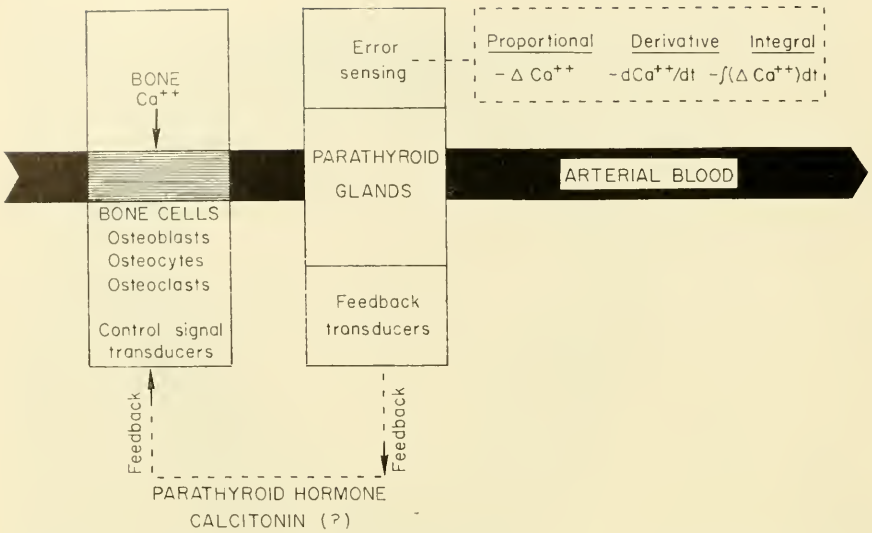

FIG. 4. Diagram of control system regulating Ca^{++} concentration in the blood. For explanation, see text.

cytes, and osteoclasts—are potentially capable of performing this function; available evidence suggests that this is done by a combination of osteoclastic resorption with release of H^+ from the glycolytic cycle of the osteocytes; there is no specific suggestion that the osteoblasts contribute to the release of calcium from bone.

In the absence of the parathyroid glands there is a decrease in the concentration of calcium in the blood plasma, with 7 mg per 100 cc as a representative figure; the transfer of calcium between bone and blood continues under these conditions. The dual mechanism of McLean and Urist attributes this to ion transfer, not under the control of the parathyroids. Osteoclastic resorption continues, as demonstrated by Jowsey *et al.* (1958); new haversian systems are formed; and the glycolytic cycle of the cells of bone doubtless continues to be active. There must remain in bone all of the mechanisms concerned in ion transfer or ion exchange; what is lost is the parathyroid mechanism, which detects and corrects any deviation from the normal concentration of Ca^{++} in the blood plasma.

One further proposal remains for consideration, i.e., the possibility that there is a change in the chemical nature of the mineral of bone,

as it matures during the mineralization of osteons. The terms labile and stable bone mineral have been in use for some time, but there has not been a chemical definition of these terms. There is, however, particularly in the work of Dallemagne *et al.* (1961), a suggestion that as bone mineral matures it may change in chemical composition, and hence in reactivity. This possibility may be explored, although any tentative conclusions must be regarded as speculative.

The basic structure of the bone mineral, as now generally accepted, is that of *hydroxyapatite,* $Ca_{10}(PO_4)_6(OH)_2$. Dallemagne, however, has for some years supported the view that the mineral, as it exists in living bone, is a *hydrated tricalcium phosphate,* $Ca_9(PO_4)_6H_2(OH)_2$ (alpha tricalcium phosphate), and has believed that when this mineral is isolated from its organic matrix, it assumes the more stable form of hydroxyapatite. Posner and Perloff (1957), however, have proposed the concept of a *calcium-deficient apatite,* and regard Dallemagne's hydrated calcium phosphate as a special case of such a mineral. Posner *et al.* (1960) propose that such a calcium-deficient apatite can be accounted for by hydrogen bonding. One example may be $Ca_9(PO_4)_4(OH)$ $(O_3PO—H—OPO_3)$.

The hypothesis is here suggested that the highly reactive form of the bone mineral, subject to rapid ion exchange at the surfaces of the crystals, may be regarded as a calcium-deficient apatite, with hydrogen bonding accounting for its crystal structure, and that as this mineral matures with aging it is transformed into the more stable hydroxyapatite.

Summary

Internal remodeling of compact bone continues throughout the life of the individual. By providing a continuing supply of reactive bone mineral, from which calcium lost from the blood in a rapid turnover of this element is replaced, this performs a function essential to life.

Internal remodeling is accomplished by formation of resorption cavities, or tunnels, in the bone, as a result of osteoclastic resorption; an analogy with rock boring by biologic organisms, as described elsewhere in this volume, is suggested.

The resorption cavities are filled in by formation of new bone in concentric layers or lamellae, insuring a constant supply of new or *metabolic bone;* this new bone takes up radioisotopes, such as Ca^{45} and Ra^{226}, preferentially, leading to concentration in *hotspots,* demonstrable by autoradiography. As metabolic bone matures it loses its reactivity, and adds to the mass of *structural bone.* It is suggested that maturation of the bone, with a decrease in its reactivity, represents a change in the chemical nature of the mineral.

The reactive bone in the newly forming osteons participates in the exchange of Ca^{++} between bone and blood; this is in part under control of the parathyroid glands, and in part independent of them; the parathyroids, however, serve to control the level of Ca^{++} in the blood plasma. It is suggested also that vitamin D may play a part in the regulation of exchange of calcium between blood and bone.

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15

Rarefying Disease of the Skeleton: Observations Dealing with Aged and Dead Bone in Patients with Osteoporosis

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THE subject of rarefying disease of bone in clinical medicine includes at least ten relatively uncommon conditions and one very common disorder. Some of the uncommon conditions are: osteomalacia, osteitis fibrosa generalisata, renal osteodystrophy, hypophosphatasia, metastatic bone disease, osteitis deformans, fibrous dysplasia of bone, multiple myeloma, scurvy, and osteogenesis imperfecta. The one common disorder is osteoporosis, a condition very difficult to diagnose at an early stage and often superimposed upon physiological atrophy of bone of old age. In the advanced stage,

osteoporosis is recognized by *bone failure* or spontaneous collapse of vertebral bodies, ballooned discs, and thin cortex in radiographs of the dorsal and lumbar regions of the spine. After a thorough clinical and laboratory study of the patient, it is easy to identify osteoporosis as a disease by these three radiographic changes. Patients with osteomalacia and hyperparathyroidism may present the same radiographic picture, but the clinical and laboratory findings are different and the number of cases is relatively small. Patients with diffuse decrease in radiographic bone density (a highly subjective observation), without any deformity of the vertebrae, are classified as examples of physiologic atrophy of bone of old age. Patients in an early stage of the disease process of osteoporosis cannot be diagnosed by clinical or laboratory methods that are now available to physicians.*

Controversy exists about whether the osteoporosis is an endocrine (metabolic) disorder, a degenerative disease, or an integral part of the time-dependent process of aging. Some investigators claim that osteoporosis is simply mild chronic osteitis fibrosa generalisata caused by prolonged calcium deficiency (Fraser, 1962); others contend that osteoporosis consists of generalized bone atrophy that is complicated by either osteitis fibrosa or osteomalacia (Clerkin *et al.*, 1962) or both (Meroney *et al.*, 1959) and, therefore, is not a discrete condition; others (Little *et al.*, 1962; Casuccio, 1962) postulate that osteoporosis is primarily a degenerative disease of bone tissue. A larger number of cases were reported in 1961–1962 than in the preceding 5 years, and more new information was obtained about pathogenesis of the disorder than in all of previous time. The etiology, however, is not known and treatment is unsatisfactory.

* Osteoporosis may be present or absent in individuals with a number of diverse conditions, as, for example, acromegaly, diabetes, hyperthyroidism, gonadal agenesis, Cushing's syndrome (endogenous and exogenous), pernicious anemia, starvation, tumors, cirrhosis, von Gierke's disease of the liver, posttraumatic metabolic syndrome, and postpregnancy state. The proportion of these diagnoses in the total number of patients with osteoporosis is 19 per cent. The proportion of osteoporosis in otherwise healthy individuals, between 50 and 75 years of age, generally classified in hospital records as postmenopausal or senile types is 81 per cent (Moon and Urist, 1962). Osteoporosis is frequently more severe in patients with than in patients without the above diseases, but the causative relation is indirect and involves poorly understood complex reactions of bone tissue.

The object of this communication is to (1) submit observations on 5-year follow-up examinations of 100 women with an average age of 85; (2) add two cases of osteoporosis to six cases previously reported and intensively studied by correlated methods; (3) analyze the literature of the period between 1961 and 1963 and some previous articles that lend support to current research work. Articles appearing between 1957 and 1961 (Urist *et al.*, 1962; Urist, 1962) and reviews of previous years have already been discussed in detail (Vincent and Urist, 1961; Gordan, 1961; Fraser, 1962; Moon and Urist, 1962).

Case Material and Results

Case No. 7

Bl. S., a childless housewife, 67 years of age, had severe backache for a period of 3 years. The condition began when she lifted a chair, experienced sharp pain in the low back, and was found to have spontaneous collapse of two vertebrae. The pain subsided after treatment with a corset and sex hormones for 9 months. One year later, she had another attack of backache associated with spontaneous fractures of the lower dorsal spine. The response to the treatment was unsatisfactory. The past history was interesting in that the patient was treated for anemia for 8 years. She lost 15 pounds and 1 inch in height in a period of 1 year. Physical examination revealed a flattening of the dorsal and lumbar spinal curves, and deep circumferential skin folds between the costal margin and iliac crests. Radiographs showed old compression fractures of the 7th, 9th, and 11th dorsal, and 1st, 2nd, and 4th lumbar vertebrae; intervertebral discs between the unfractured segments were ballooned and the cortex was everywhere thin (Figs. 1A to 1C). The results of laboratory studies were as follows: sternal marrow cytology was negative for myeloma cells; hemoglobin, 11.0 gm %; sedimentation rate, 40 mm/hr. (Wintrobe); white blood cells and differential counts were normal; serum protein 6.9, albumin 3.7, and globulin 3.2 gm % with A/G ratio of 1.1; protein-bound iodine, 5.7 mg %; serologic tests for syphilis were negative; cholesterol, 262 mg %; serum calcium, 11.0 mg %; inorganic phosphorus, 3.5 mg %; alkaline phosphatase, 8.2

FIG. 1A. Lateral-view radiograph of dorsal spine (Case No. 7, Bl. S.) shows bone failure or collapse of 7th, 9th, and 11th dorsal to 1st lumbar vertebrae.

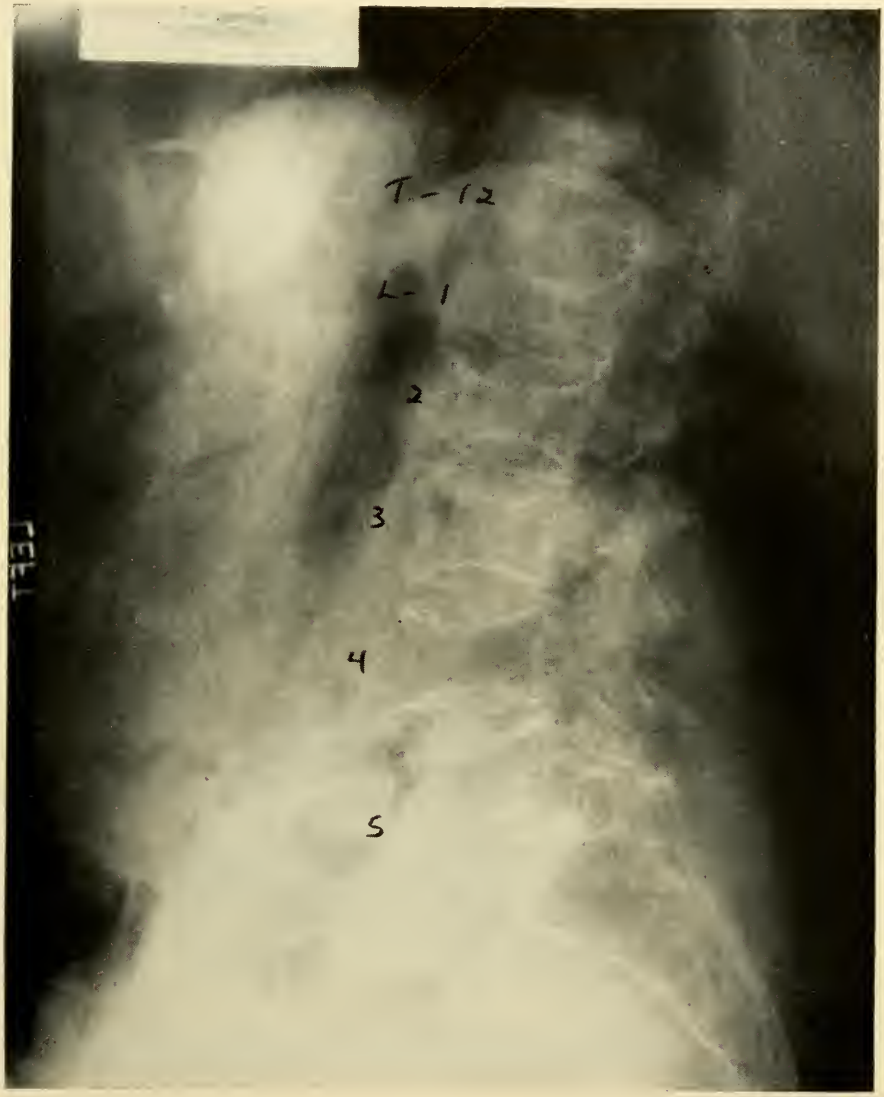

FIG. 1B. Lateral-view radiograph of lumbar spine in case shown in Fig. 1A, showing thin cortex, ballooned discs, and bone failure in the 1st, 2nd, and 4th lumbar vertebrae.

FIG. 1C. Snapshot showing the patient (Case No. 7) performing bicycling exercises. Note the foreshortened trunk, due to collapsed vertebral bodies in the spine.

King Armstrong units; serum citric acid, 2.9 mg %; quantitative determinations for fat in the stool were within normal limits; routine urinalysis presented no abnormalities.

PROLINE TOLERANCE TEST. The rate of bone formation in Case No. 7, as compared with normal growing children and an osteoporotic patient with a fracture of the hip, was investigated by means of

the proline tolerance test of Summer (1961) and Ibsen *et al.* (1963). In this test the rate of disappearance of proline from the blood was increased during the period of rapid formation of callus and new bone. In Case No. 7, the rate was the same as in normal nonosteoporotic subjects, but lower than in young individuals and osteoporotics during a period of callus formation (Fig. 2).

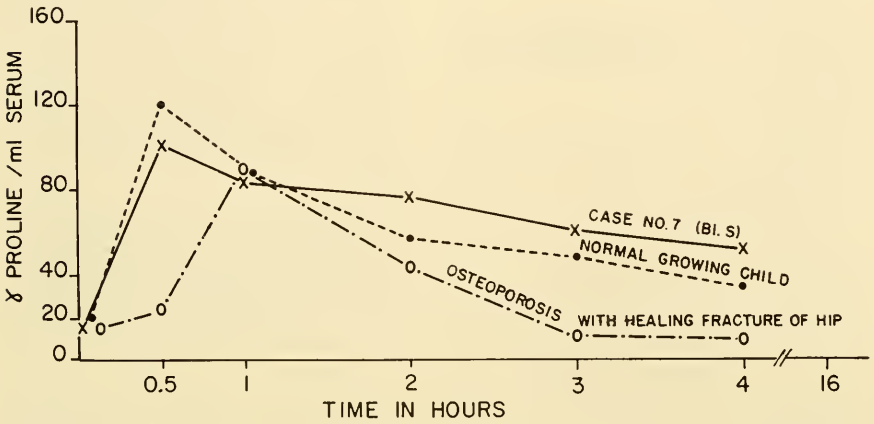

FIG. 2. Graph showing results of proline tolerance test in Case No. 7, showing normal bone formation rate as determined by the rate of disappearance of an intravenously injected dose of 65 mg proline per kg body weight.

METABOLIC BALANCE. During a control period of 20 days, Case No. 7 was in negative nitrogen, phosphorus, and calcium balance. This was restored to equilibrium with respect to nitrogen and phosphorus, but positive calcium balance developed during a period of 15 days of treatment with oral administration of 65 mg per kg of proline per day. In order to determine whether the effect was upon intestinal absorption, 500 mg of proline was administered daily by intramuscular injection for an additional 15 days; the patient remained in nitrogen and phosphorus equilibrium but returned to negative calcium balance. Proline was empirically selected for this study because it is a precursor to collagen, known from animal experiments with proline-H^3 to be deposited in bone matrix (Fig. 3).

RADIOISOTOPE KINETICS. Figure 4 illustrates the excretion of an injection of 5 μc of Sr^{85}, as determined by the changes in the amount

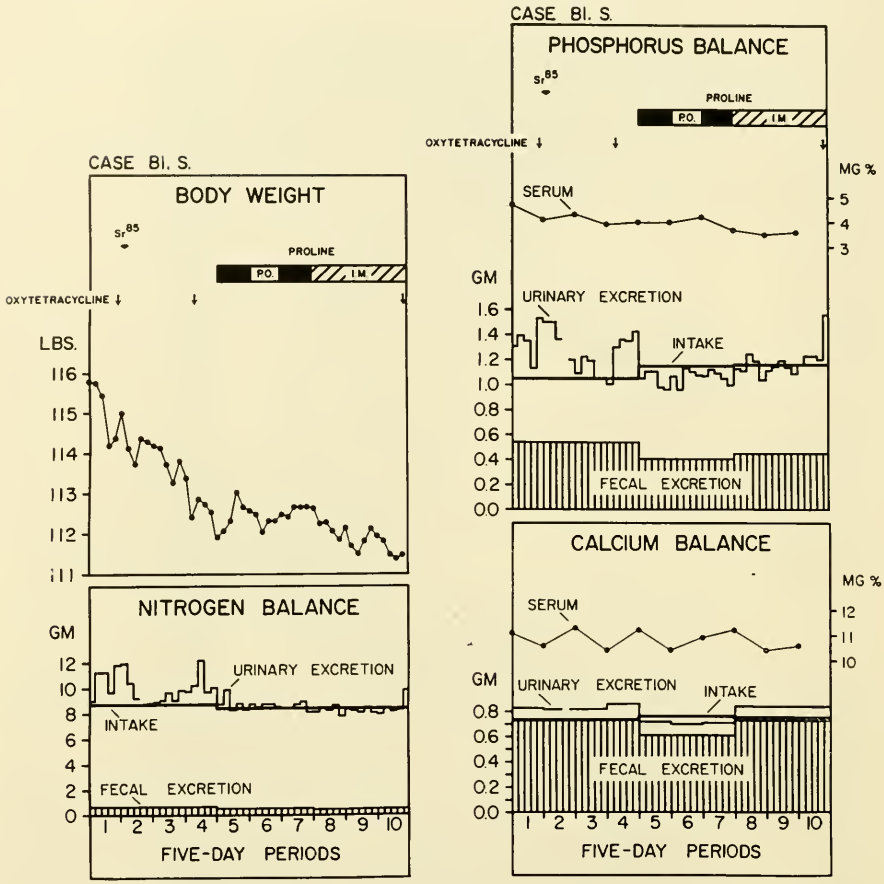

FIG. 3. Metabolic balance studies for nitrogen, calcium, and phosphorus in Case No. 7, showing loss of nitrogen, phosphorus, and calcium in the pretreatment period, restoration of equilibrium during oral administration of proline, and return to negative calcium balance during treatment with intramuscular injections of proline.

detected by means of whole body counting in Case No. 7. The close correspondence between the results of excretion as determined by retention in the skeleton and assay of absolute amounts of Sr^{85} in the urine and feces demonstrated that the use of a whole body counting facility can eliminate the costly and more time-consuming balance studies for calcium in the future. The osteogram and Bauer-

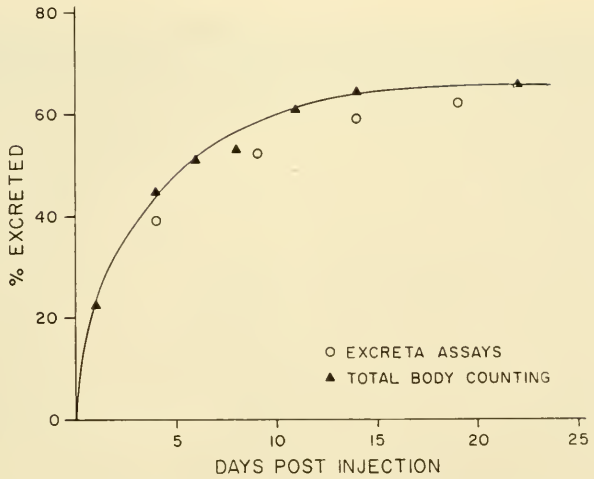

FIG. 4. Graph showing the rate of excretion of Sr^{85} in Case No. 7, as measured both by the percentage of the dose detected by the total body counting technic, and by direct radio assay of total urinary and fecal output. The results of the two methods are entirely comparable and equally accurate.

Carlssen-Lindquist calculations were not completed in this case because of mechanical failure of the counting equipment during the period of the balance study.

TETRACYCLINE LABELING AND BIOPSY. Oxytetracycline was infused intravenously in a dose of 1.0 gm per liter of 0.9 per cent solution on the 5th and 20th days of the control period and again on the 50th day. A biopsy was performed on the upper end of the tibia at the conclusion of this study on the 50th day. The thickness of the cortex, measured with the aid of a micrometer, was 0.6 mm or 50 per cent of that of young adult women. On this basis, assuming that the thickness of the cortex of the upper tibia reflects the density of the whole skeletal system (Lindahl and Lindgren, 1962), the skeleton in this patient weighed approximately 5.6 kg. The spaces between the tetracycline lines, considering the 30- versus the 15-day interval, indicate no increase in bone formation rate (Fig. 5A).

On the histological level, in the low-power magnification with the light microscope, the bone that persists (in Case No. 7) was laid down in the normal configuration and had the appearance of normal

FIG. 5A. Fluorophotomicrograph showing triple labeling of cortical bone in Case No. 7. The space between the two outer rings reflects the amount of bone deposited during a 15-day control period, and the space between the middle and inner ring indicates the amount deposited in a 30-day period on treatment with proline. With due allowance for the well known fact that the bone deposition rate is high at the beginning and low at the end of the period of formation of a new osteon, the longer interval and wider space indicate that the treatment did not increase the mean osteon formation time for the bone in the upper end of the tibia. (× approx. 450.)

bone. In high-power magnification, however, some parts of the skeleton presented an abnormal change in that they had large areas in which the lacunae had lost their osteocytes. The cell-free areas were frequently in the outer layers of the osteons and in the interstitial lamellae of cortical bone. The matrix also stained irregularly basophilic and metachromatic rather than as a homogeneous eosinophilic structure. Occasionally, it was possible to see strips of tissue in which the collagen fiber bundles accepted the Wilder stain irregularly. In some areas of the surface compacta, as, for example, in the neck of the femur and the anterior cortex of the vertebral bodies

(in autopsy subjects), there were patches of basophilic metachromatic, acellular necrotic bone that predisposed the patient to a spontaneous fracture.

The above changes also occurred in nonosteoporotic bone from both men and women of comparable age, but quantitatively the degree was significantly less than in osteoporotic subjects (Table VI). The osteocytes in inner lamellae around blood vessels averaged 6.5 cells per high-power field in nonosteoporotics and 2.7 in osteoporotics. In the outer lamellae, the outstanding difference was in the number of retracting osteocytes, 6.0 and 3.7, and calcified osteocytes, 3.0 and 1.5, in osteoporotics and nonosteoporotics respectively. In the interstitial lamellae, the average number of enlarged empty lacunae was 28.2, compared with 22.0. To judge from the appearance of increasing amounts of bone with empty lacunae with increasing age and with chronic peripheral vascular disease, it was assumed that this condition represents devitalized bone from prolonged circulatory insufficiency (Sherman and Selakovitch, 1957). We have reviewed the biopsy sections of our previously reported cases (Urist *et al.*, 1962) and observed that osteocytes move to one side and then enlarge the lacuna by absorption of matrix between the canaliculi, possibly through the action of a proteolytic enzyme. Later, the cell undergoes autolysis, and an empty enlarged spherical (rather than stellate and ovoid) lacuna remains. These morphologic changes were unspecific but approximately 30 per cent more extreme during middle-aged life in patients with severe osteoporosis (Figs. 5B to 5I).

Quantitative analyses of oxytetracycline in the biopsy sample by the method of Ibsen *et al.* (1963) revealed 4 γ per gm of bone. This represented the amount deposited in the skeleton in Case No. 7 after infusion of 3.0 gm in a period of 45 days. This was 3.2 γ less than the amount to be expected, inasmuch as the average nonosteoporotic deposited 2.4 γ after one infusion of 1.0 gm. Fluorescent microscopy demonstrated that very few osteons, approximately 5 per cent of the total number in the section, were growing and removing oxytetracycline from the extracellular fluid; in control subjects of comparable age, approximately 10 per cent were reactive. Examination of the active osteons by white light microscopy re-

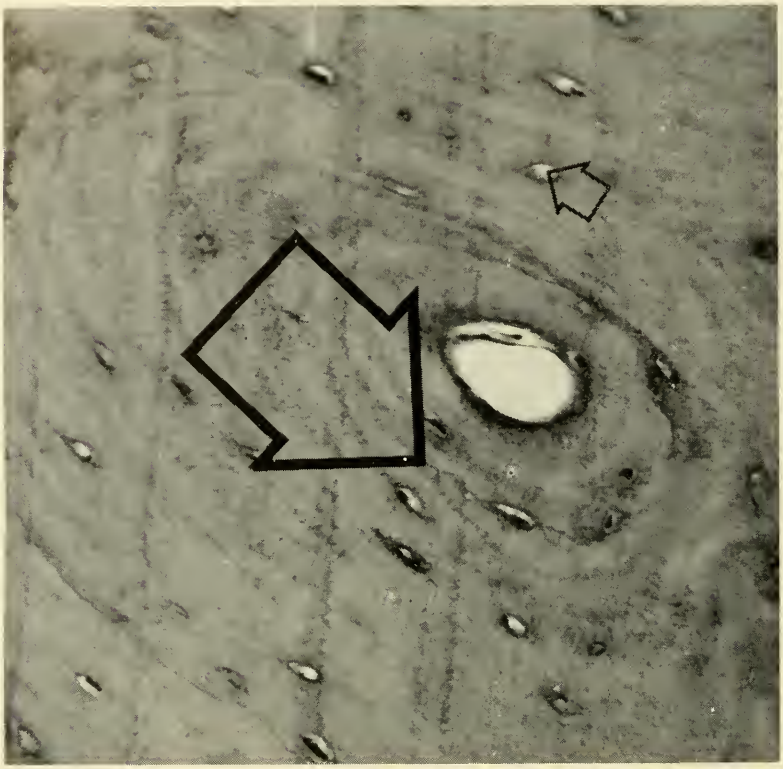

FIG. 5B. Photomicrograph showing a typical osteon in cortical bone in osteoporosis. The osteocytes in the inner lamellae near vessels appear normal, whereas osteocytes in the outer lamellae (large arrow) are retracting. The lacunae in the interstitial lamellae between osteons are enlarged and transformed from a thin stellate to a spherical cavity either containing a pyknotic nucleus or entirely empty (small arrow). Hematoxylin, eosin, and azure II stain. (× approx. 450.)

vealed normal stellate osteocytes in both the inner and outer lamellae of the growing osteons. Nearly *all* the nongrowing osteons had retracting, enlarged, or devitalized osteocytes in the outer lamellae (Fig. 5C) and empty lacunae in the interstitial lamellae. There was no way of measuring the age of the inactive osteons (those with a hypercalcified inner margin), but the oxytetracycline label at three intervals suggested that approximately 12 weeks was the time that

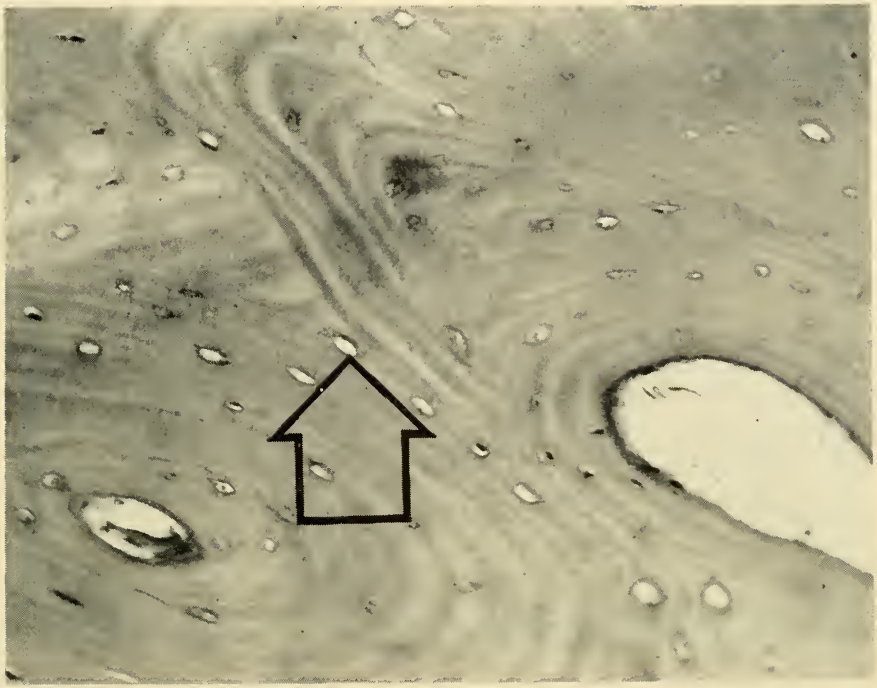

FIG. 5C. Photomicrograph showing a typical area of osteoporotic interstitial bone. The lacunae are enlarged, spherical, smooth-walled, and empty (arrow). The broad lines between the lamellae represent thick layers of metachromatic-staining cement substance, presumably deposited on exposed surfaces of matrix under conditions in which the rate of bone formation is abnormally low. (× approx. 400.)

an osteoporotic required to build a new osteon. However, many osteons stopped growing and developed a sclerotic inner margin before they were three-fourths closed in aged and osteoporotic individuals.

The life expectancy of an osteocyte in an outer lamella (the area of the osteon farthest from the central blood vessel) in aged persons with osteoporosis and chronic circulatory insufficiency is probably not more than 12 weeks. Further investigations with the aid of continuous administration of oxytetracycline are in progress to measure this interval in both nonosteoporotic and osteoporotic subjects.

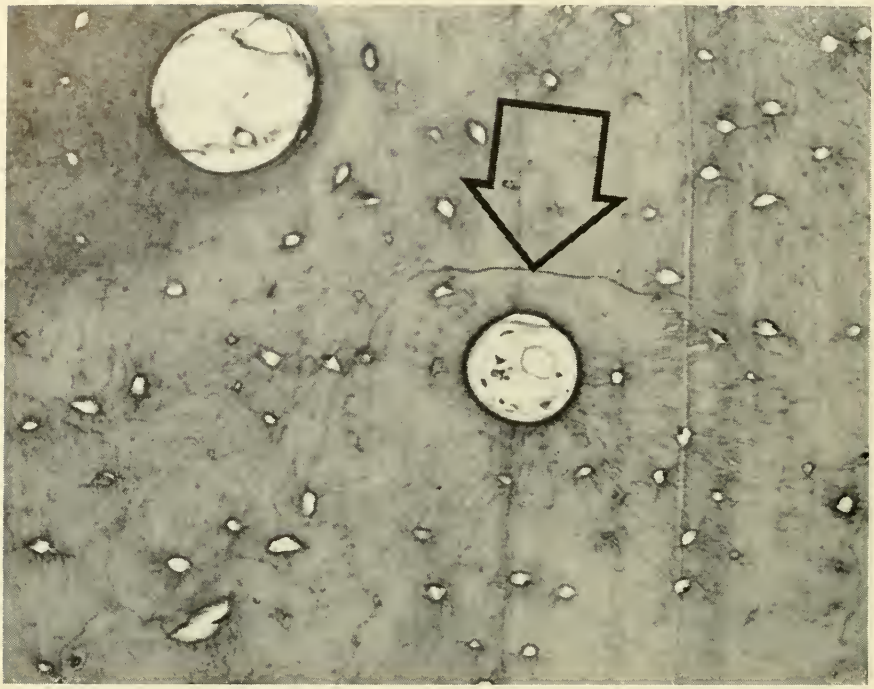

FIG. 5D. Photomicrograph showing an area of devitalized bone in the center of the cortex of the tibia in severe osteoporosis. The lacunae are enlarged everywhere and the osteocytes are either pyknotic, fragmented, or absent. The thin line indicated by the arrow represents a rapidly deposited metachromatic-staining cement substance on the outer wall of a resorption cavity; this is the reversal line that marks the beginning of construction of a new osteon. (× approx. 400.)

Case No. 8

M. R., a retired school teacher, 71 years of age, had severe backaches, pain in both lower extremities, and was unable to walk without the aid of two canes. At the age of 68, while lifting an empty bookcase weighing less than 25 pounds, she incurred a compression fracture of the 12th dorsal vertebra. One month later, three additional vertebrae, the 9th and 10th dorsal and the 1st lumbar vertebra, collapsed following a trivial injury. She lost 6 cm in height and 25 pounds in weight in a period of 6 months (Figs. 6A and 6B). The treatment of the fractures consisted simply of bed rest and 5 mg

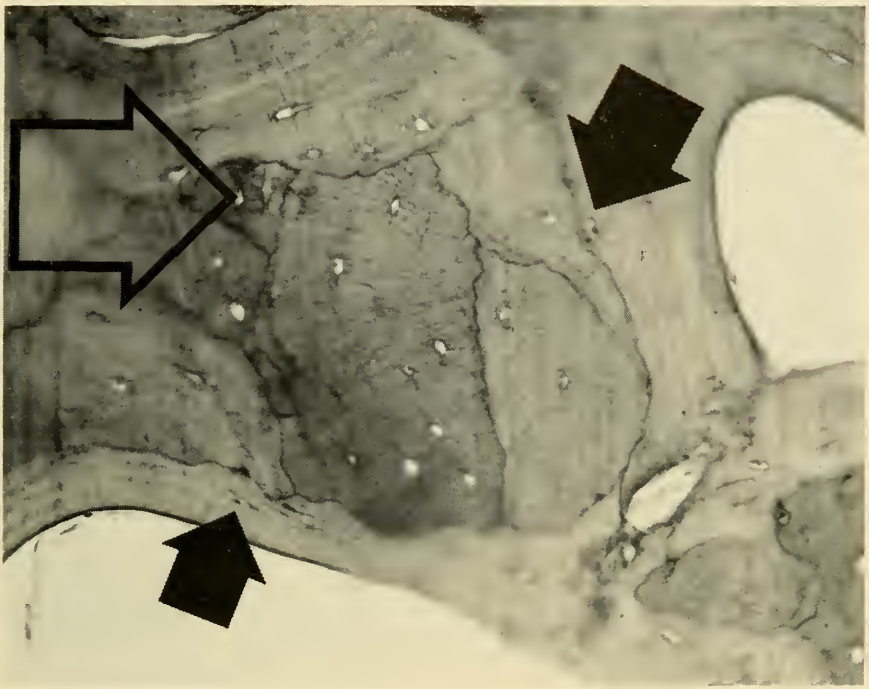

FIG. 5E. Photomicrograph illustrating a complex of six areas of interstitial bone enclosed in thin reversal cement lines between two half-closed osteons. The matrix of old bone surrounds enlarged empty lacunae and is strongly basophilic and metachromatic rather than eosinophilic (open arrow). Only the new bone surrounding large vascular channels contains small living osteocytes (indicated by black arrows). (× approx. 400.)

of methandrostenolone (Dianabol) per day for 6 weeks. She regained most of the weight loss, but the backache persisted. The past history was remarkable in that the patient was treated for hyperthyroidism, gastroenteritis, and herpes zoster when she was 66 years of age. The diagnosis of osteoporosis was not considered because, during the 7 months preceding the first fracture, she received small doses of ACTH and triamcinolone empirically for treatment of vague bone and joint pains. This was discontinued as soon as radiographs of the spine revealed collapsed vertebrae (Figs. 6C to 6E). The calcium intake was estimated at 700 mg per day, and her food habits in general were evaluated as normal. Routine hospital labora-

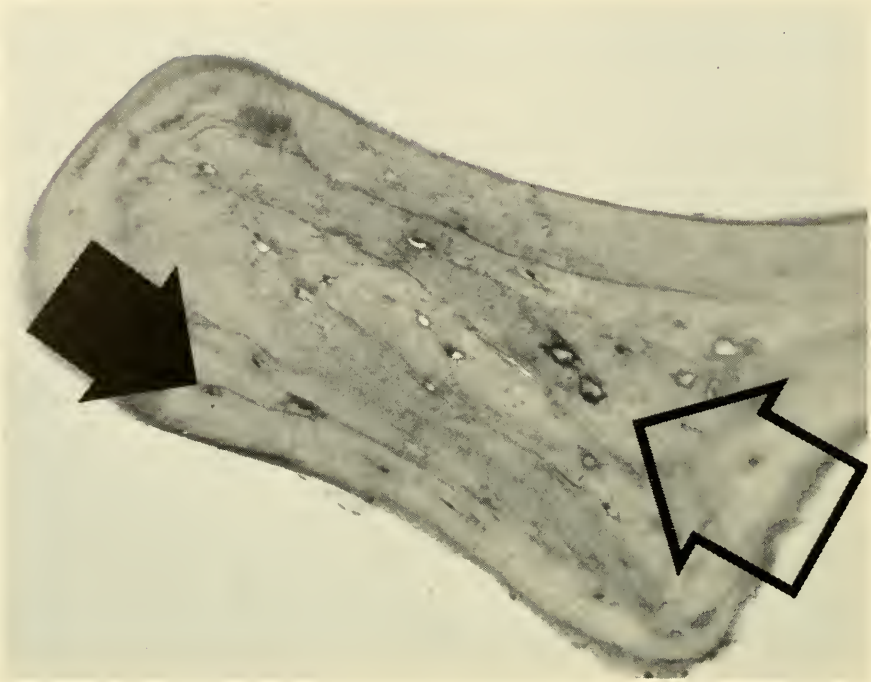

FIG. 5F. Photomicrograph showing trabecular bone in severe osteoporosis. The total number of trabeculae is low, and some are attenuated, but a few are decreased in length and increased in thickness. The enlarging empty lacunae and shrinking osteocytes are in the core (open arrow), while the living osteocytes (black arrow) are on the surface. (× approx. 400.)

tory studies showed no abnormality. The laboratory tests given Case No. 7 were also given Case No. 8, and the results were all within the limits of normal.

METABOLIC BALANCE STUDY. During a control period of 15 days, the patient complained of backache and spent 20 hours a day in bed. She lost weight and was in negative calcium, phosphorus, and nitrogen balance. Polysaccharides (Dingwall *et al.*, 1962; Westcott *et al.*, 1962) administered by intramuscular injection, 5 mg per day, between the 15th and 30th days, did not prevent further decline in weight, or change the calcium, phosphorus, or nitrogen balance. Human growth hormone (supplied by the Endocrine Study Section,

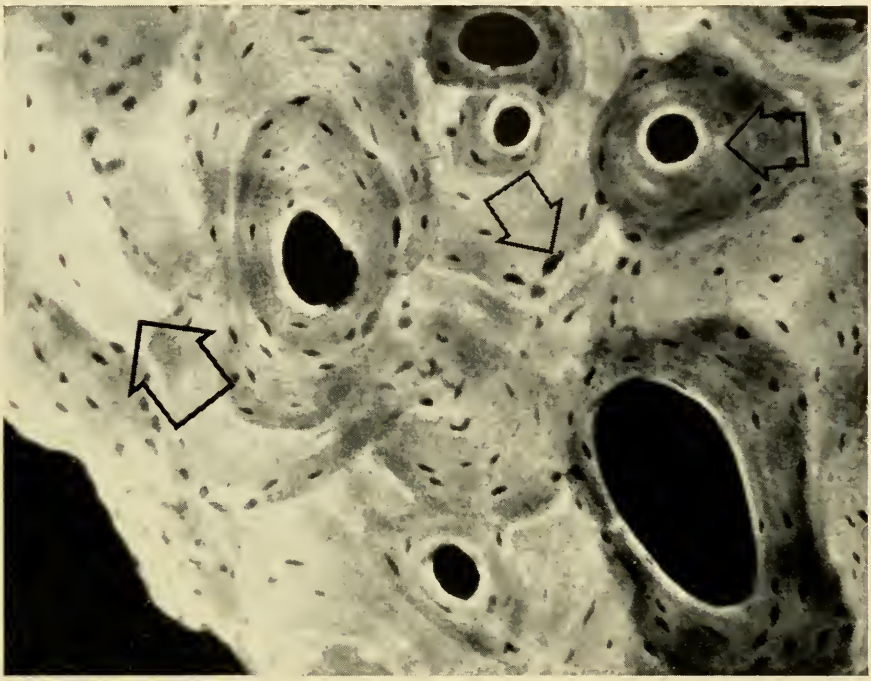

FIG. 5G. Microradiograph of the endosteal surface of the cortex showing old bone in a low-density osteon with a sclerotic inner wall and calcified osteocytes (arrow on the right). The arrow near the center indicates an enlarged lacuna with a rarefied zone of matrix around it. The arrow on the left points to hypermineralized interstitial bone in which many of the lacunae are filled with calcium salts. The osteon with the large vascular channel in the lower right corner of the picture has a sclerotic inner ring; this occurs when bone formation ceases before the system is half closed; quantitatively, this appears more frequently in osteoporotics than in nonosteoporotics and causes increased porosity of the cortex. (× approx. 400.)

National Institutes of Health, United States Public Health Service, 1962) was administered by intramuscular injection of 2 mg per day from the 50th to the 65th day; this produced 2.0 gm of positive nitrogen balance but little or no improvement in the negative calcium and phosphorus balance; the urinary calcium excretion, normally only 50 to 100 mg per day in aged osteoporotics, increased from 100 to 200 mg per day. These effects of growth hormone were associated with further decline in weight, and probably did not

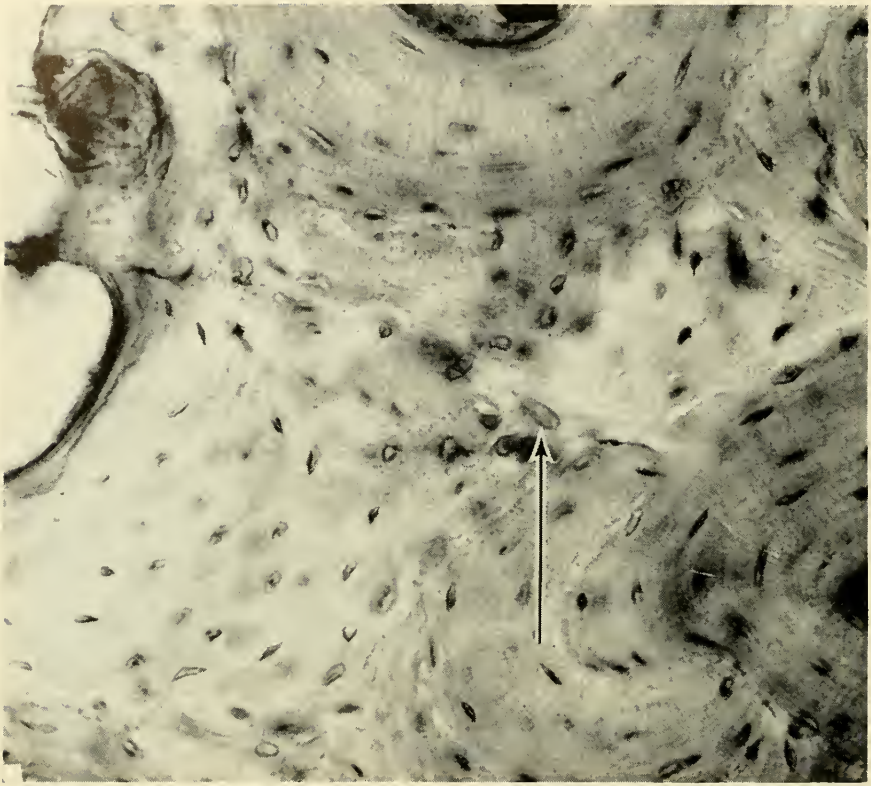

FIG. 5H. Photomicrograph illustrating an enlarged lacuna in hyperminer-alized interstitial bone (arrow). This cell is also shown in Fig. 5 I. Osteocytes with normal nuclei and lacunae of normal shape and size are found only in perivascular lamellar bone. (× approx. 400.)

reflect specific action upon either the skeletal system or the osteo-porosis (Fig. 7).

RADIOISOTOPE KINETIC STUDY. An intravenous injection contain-ing 5 μc of Ca^{47}, 5 μc of Sr^{85}, and 1 μc of I^{131} labeled serum albumin was administered during the period of treatment with daily injec-tions of polysaccharides extracted from bone, and again during the period of treatment with human growth hormone. Osteograms of the midshaft of the tibia during polysaccharide treatment showed a slow change in rate of uptake, rising over a period of more than

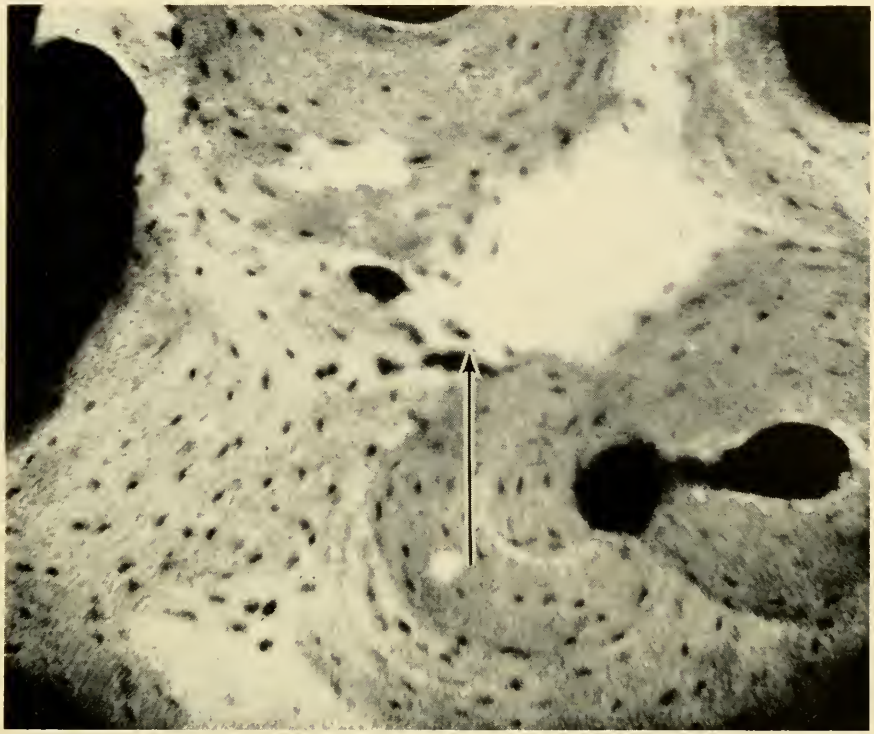

FIG. 5 I. Microradiograph showing the identical area and osteocyte lacuna (see arrow) illustrated in Fig. 5H. Note the hypermineralized interstitial bone and the low-density mineralized lamellar bone in the lower right osteon. The sclerotic inner margin suggests that this osteon represents a deposit of old bone. (× approx. 400.)

1 hour; on treatment with human growth hormone, the rate of accumulation was the same as in normal subjects, where the accumulation of the radioisotopes reached a plateau in 15 minutes. Other areas of the skeleton responded in the same way as the tibia (Fig. 8). The daily loss of Sr^{85} in the urine during treatment with polysaccharide, especially during the first 3 days, was less than with human growth hormone. One-half of the Sr^{85} was excreted in 5 days when the patient received polysaccharides; on human growth hormone, 50 per cent was excreted in only 2 days (Fig. 9). Ca^{47} was much better retained in the skeleton than Sr^{85}. The 50 per cent excretion point was reached after 18 days on polysaccharide treatment but only 5 days on human growth hormone. This clearly

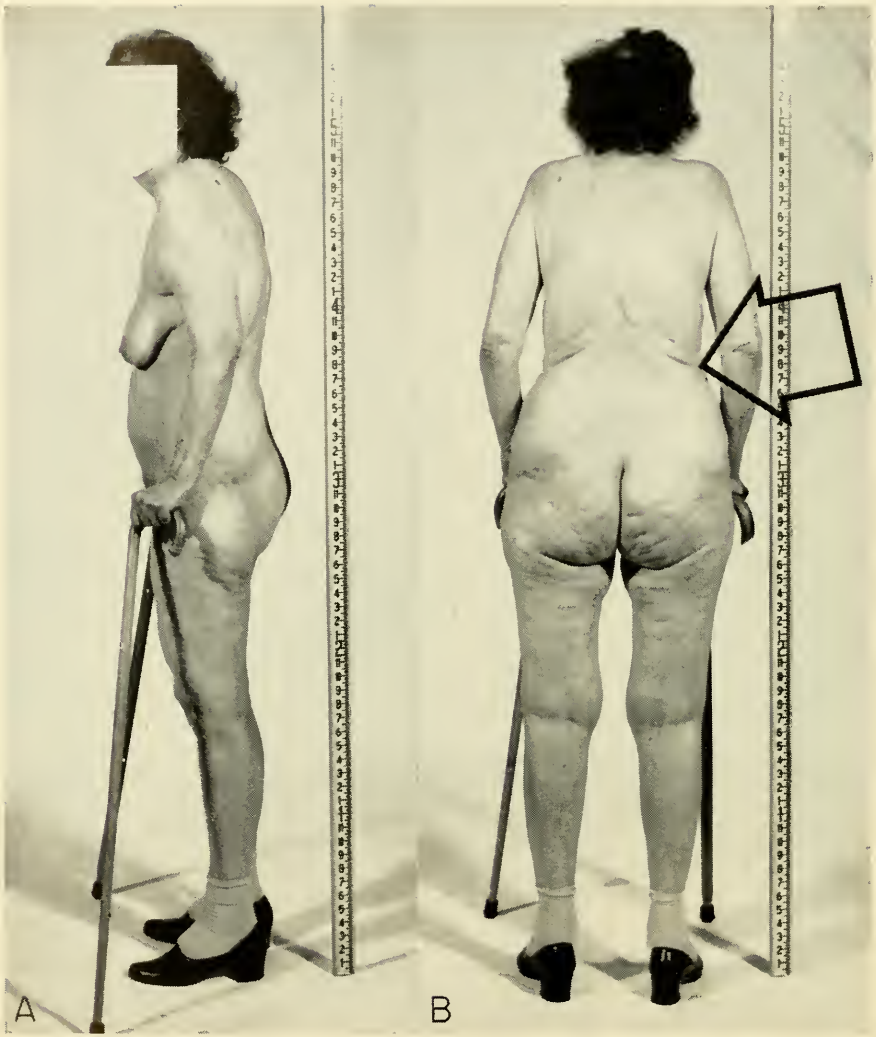

FIG. 6A. Photograph of 71-year-old retired school teacher (Case No. 8, M. R.) showing fixed flexion of the cervical spine and flattening of the dorsal and lumbar spine. This patient lost 6 cm in height.

FIG. 6B. View of the back (Case No. 8) illustrating skin folds (arrow) produced by telescoping of the torso.

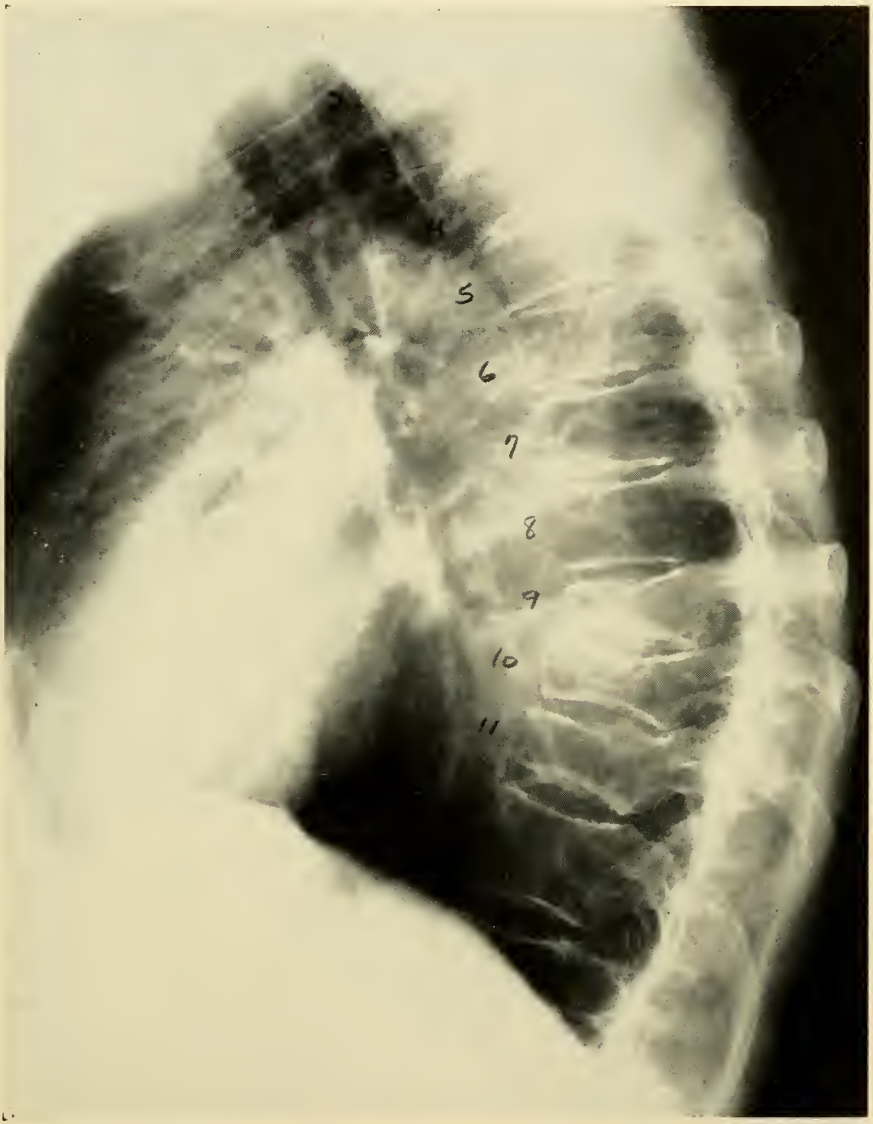

FIG. 6C. Lateral-view radiograph of the dorsal spine, showing bone failure and collapse of the 9th to the 11th dorsal vertebrae (Case No. 8).

FIG. 6D. Lateral-view radiograph of the lumbar spine of the case shown in Fig. 6C, illustrating ballooned discs, thin cortex, and collapse or bone failure of the 3rd lumbar vertebra.

FIG. 6E. Anteroposterior view of the pelvis showing accentuation of the trabeculae of the internal architecture, and decrease in the density of the lateral cortex of the neck of the femur in the case shown in Figs. 6A to 6D.

demonstrated the established fact that strontium is not reabsorbed as efficiently as calcium by the kidney tubules (Fig. 10). The relative values of the exchangeable pool and calcium excretion rate, as shown in Table I, are calculated on the basis of urinary clearance of strontium, and therefore are not absolute values but can be used to compare one case with another. Our impression was that the size of the exchangeable calcium pool (on the basis of the osteogram) was the same during the entire period of treatment, but the accretion rate was greater with polysaccharide than with human growth hormone. Calcium balance was unchanged, however, and it is necessary to assume that the higher rate of accretion was associated with a higher rate of resorption. This is reflected in the urinary excretion curves of Fig. 10. The daily loss of Sr^{85} in the urine accompanying

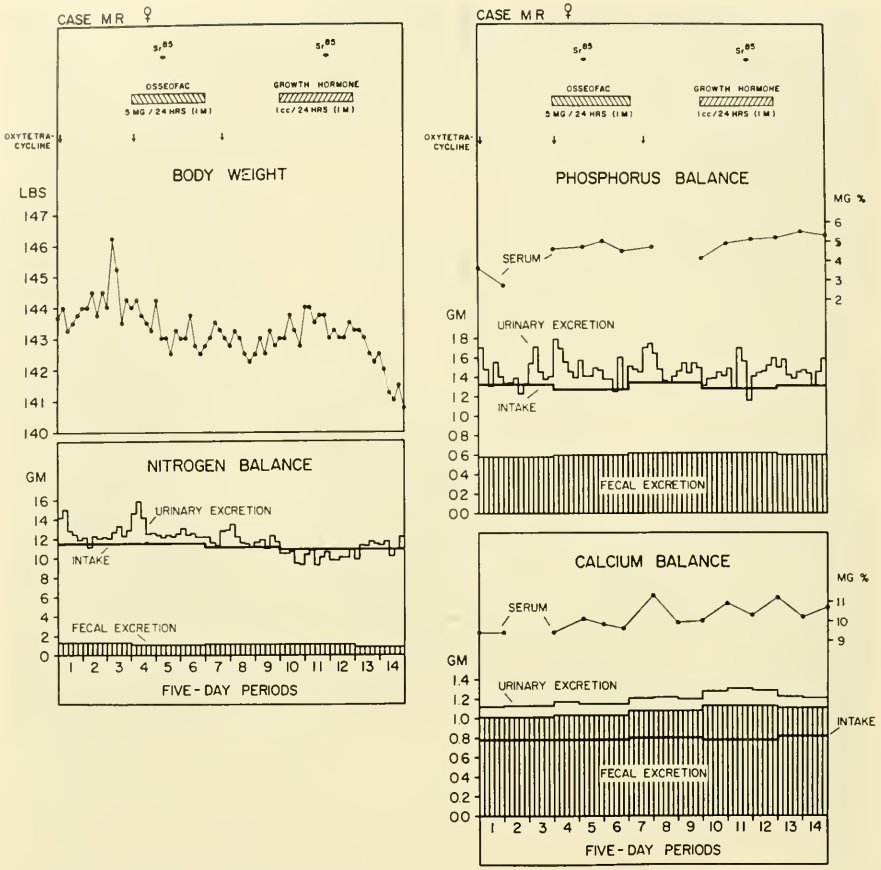

FIG. 7. Charts showing metabolic balance studies in Case No. 8. This patient lost weight and was in continuous negative calcium, phosphorus, and nitrogen balance. Polysaccharide treatment (Osseofac, Squibb) did not restore equilibrium. Human growth hormone produced nitrogen retention but did not correct negative calcium and phosphorus balances.

polysaccharide treatment was less during the first 10 days than with the growth hormone. Thereafter, *more* Sr^{85} was lost per day during the polysaccharide regime, suggesting a higher rate of resorption of bone which had been labeled with the isotope. Thus, the Sr^{85} excreted during the early days comes mainly from soft tissue and from Sr^{85} deposited on preexisting bone salt crystals by exchange rather than by incorporation into newly forming crystals (accretion). At

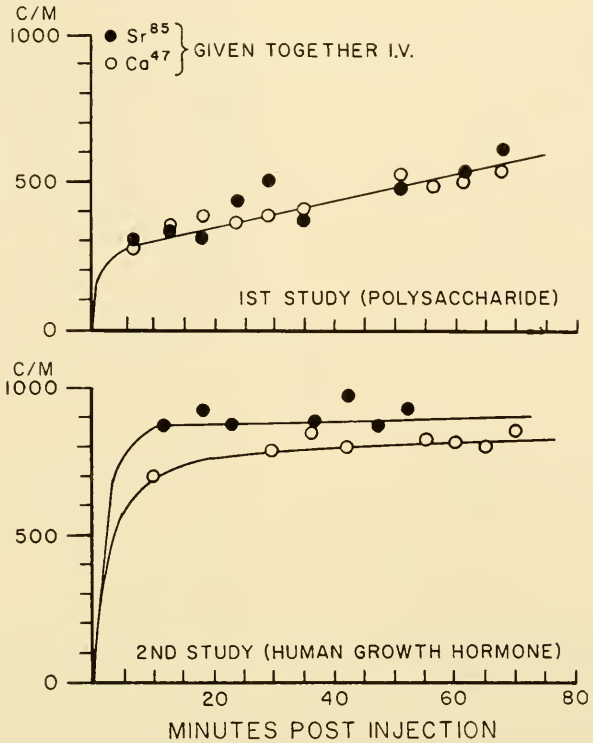

FIG. 8. Radioisotope osteogram of MacDonald (1960) showing the changes in rate of uptake of Sr^{85} and Ca^{47} in Case No. 8. The upper curve (first study) represents the reaction of the tibia during a period of treatment with intramuscular injections of polysaccharides; this suggests a high rate of deposition of bone mineral. The lower curve (second study) represents the response to treatment with growth hormone; this is the same as obtained in untreated nonosteoporotic subjects by MacDonald.

later times, however, the daily loss probably is derived primarily from continual resorption of mineral crystals which contained Sr^{85} laid down earlier by accretion.

BONE BIOPSY AND TETRACYCLINE UPTAKE. Oxytetracycline was infused intravenously, in doses of 1.0 gm per liter of 0.9 per cent salt solutions, on the 1st, 16th, and 39th days. A biopsy was performed on the 42nd day on the proximal end of the shaft of the tibia. Fluorescent microscopy revealed a triple line of fluorescence. The amount

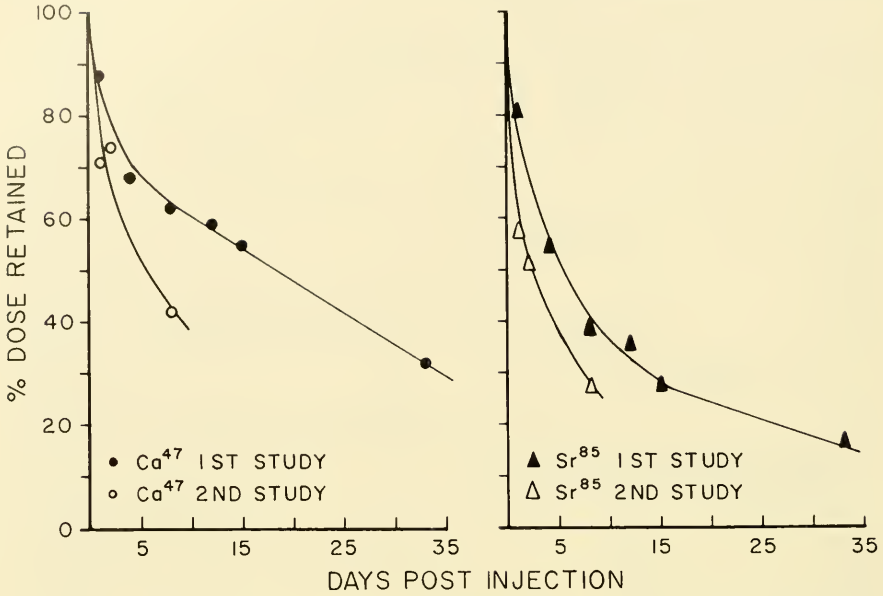

FIG. 9. Charts illustrating the retained amounts of Sr^{85} and Ca^{47} as measured by whole body counting in Case No. 8. See Fig. 8. At all times, the amounts of both isotopes retained by the skeleton were greater during treatment with polysaccharide (first study) than with human growth hormone (second study). The time required to lose 50 per cent of the Sr^{85} was 5 days for the first study, as compared with 2 days for the second.

of bone between the two inner lines represented the amount of bone deposited during the period of 15 days of treatment with polysaccharide; the amount between the middle and outer lines, the control period of 15 days of observation preceding treatment. The distance between the two lines was approximately equal. In non-osteoporotic control cases, the rate of bone formation decreased and the space between the outer and inner lines diminished in the process of closure of a labeled osteon. There was, however, considerable variation between individual osteons. Some showed the diminishing space between lines, and, therefore, no conclusive evidence of stimulation of bone formation (Fig. 11).

The histophysiologic changes observed in Case No. 7 were even more extreme in Case No. 8. All sections showed many empty lacu-

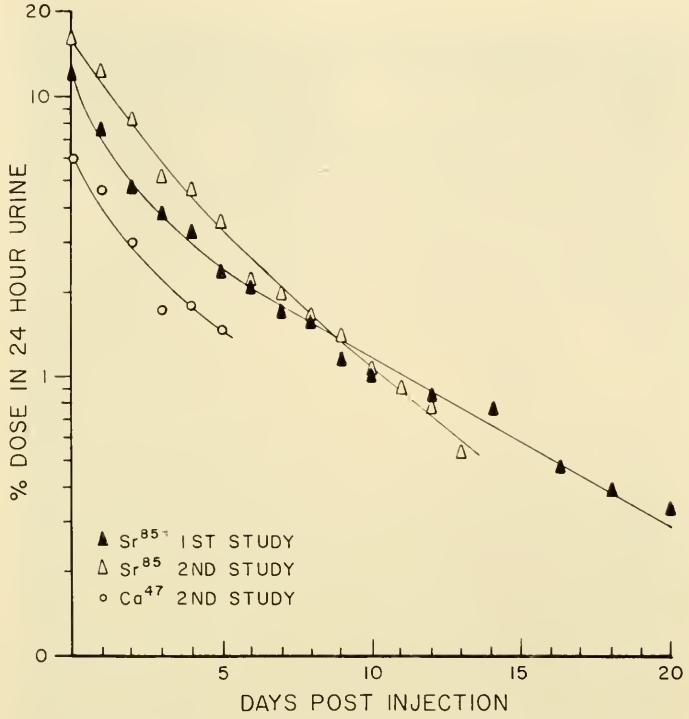

FIG. 10. Chart showing urinary excretion of Sr^{85} and Ca^{47} in Case No. 8. See Fig. 8. The total loss of Sr^{85} isotope during the first 3 days was only 23 per cent on polysaccharide therapy (first study), as compared with 37 per cent on human growth hormone (second study). These results suggest more accretion of bone salts after treatment with polysaccharide, as compared with osteoporotics treated with human growth hormone, or osteoporotics observed without treatment of any kind. However, an enhanced resorption rate is tending to counteract the increased accretion, so the net effect over a period of several weeks is small.

nae and irregular staining of matrix of both haversian and interstitial lamellae (Figs. 5B to 5G). After infusion of 3.0 gm of oxytetracycline, only a rare osteon fluoresced, and the total uptake was only 1.0 γ per gm of bone, or 84 per cent less than in nonosteoporotic controls. The over-all impression gained from these observations was that in Case No. 8 as much as 75 per cent of the bone in the tibia was dead!

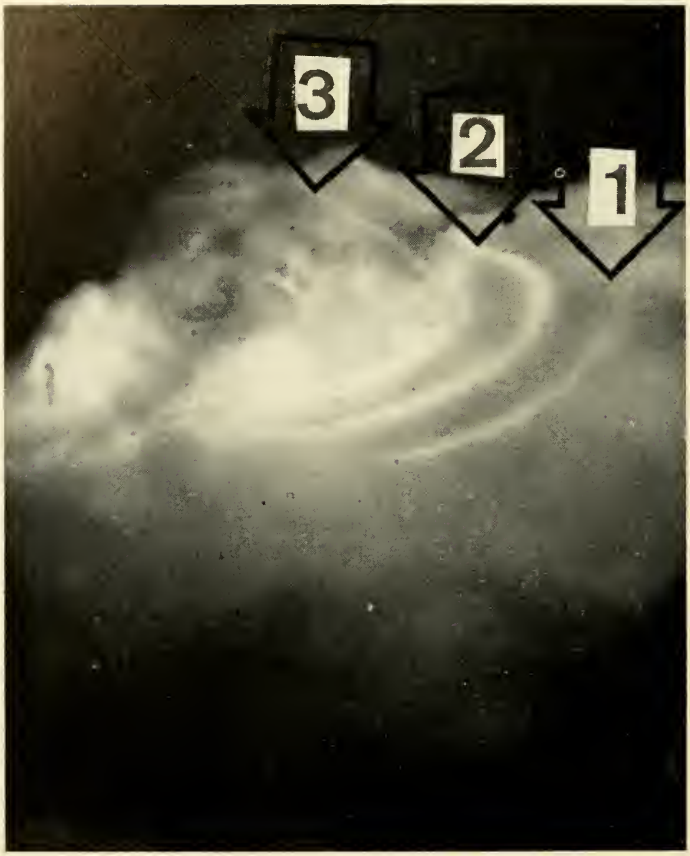

FIG. 11. Fluorophotomicrograph of undecalcified section of the proximal end of the tibia in Case No. 8, illustrating triple labeling of an obliquely cut new osteon on the endosteal surface of the cortex. The space between the two outer lines (*1* and 2) represents the amount of bone deposited during 15 days on polysaccharide therapy, and the two inner lines (*2* and 3) the control period off therapy. The equal spacing, with due consideration for the diminishing rate of deposition of bone, and the mean osteon formation time in this area of the skeleton, suggests a slight beneficial effect of treatment on osteogenesis in this case.

TABLE I. Case No. 8, M. R.: Intravenous Injections of a Mixture of Sr^{85} and Ca^{47}

Treatment	Polysaccharide (Osseofac)	Human growth hormone
Tibia osteogram	Both Ca^{47} and Sr^{85} still rising after 70 minutes	Both Ca^{47} and Sr^{85} leveled off after 15 minutes
Knee osteogram	Both Ca^{47} and Sr^{85} still rising after 70 minutes	Both rising after 70 minutes but less steeply
Urinary excretion (3 days)	Sr, 23%; Ca, lost	Sr, 37%; Ca, 14%
Total excretion (total body counting): time to lose 1st 50% of dose	Sr, 5 days; Ca, 18 days	Sr, 2 days; Ca, 5 days
Exchangeable calcium pool[a]		
From Sr^{85} data	1.9 gm	1.5 gm
From Ca^{47} data	—	5.9 gm
Accretion rate for calcium[a]		
From Sr^{85} data	0.06 gm/day	0.04 gm/day
From Ca^{47} data	—	0.22 gm/day

[a] Calculated by method of Bauer *et al.* (1961).

Summary of Correlated Observations in Cases 7 and 8, and Previous Six Cases of Severe Osteoporosis

Table II includes previously tabulated data (Urist *et al.*, 1962) and data obtained from Cases Nos. 7 and 8. This table demonstrates that correlation of the results of physiologic and morphologic methods of investigation was extremely valuable. The information gained from one method tested the validity of the other. Cases Nos. 7 and 8 (while receiving approximately the same amount of calcium as there was in the diet to which they were accustomed) were in negative calcium, phosphorus, and nitrogen balance. Case No. 7 was restored to equilibrium or positive calcium balance by proline, but tetracycline labeling showed no improvement in bone formation rate or alteration of the course of osteoporosis. In Case No. 8, balance studies showed no improvement in calcium retention, while the radioisotope osteograms revealed high or normal rates of uptake of Sr^{85} and Ca^{47} on polysaccharide therapy. The accretion rate calculated by the Bauer *et al.* (1961) formulations was also higher. The tetracycline uptake in new bone was approximately the same

as in nonosteoporotic subjects of comparable age. Judging from the extraordinary decrease in the thickness of the cortical bone, and assuming an equal loss of cancellous bone, it was apparent that both patients had lost approximately half of the mass of the skeleton. But in the face of this great deficiency, balance studies demonstrated that osteoporotics were able to turn over almost as much calcium

TABLE II. SUMMARY OF OBSERVATIONS

Case no.	Ca intake	Ca balance	$Sr^{85}O$	$Ca^{47}A$	Cx	SM	TC
C*	0.8	±25	25	0.80	100	11.6	2.4
1	0.7	+50	50	0.72	50	5.6	2.0
2	0.8	−180	21	0.19	66	7.3	4.1
3	0.5	−100	6	0.50	66	7.3	3.5
4	1.0	+5	23	0.75	50	5.6	2.4
5	0.5	−5	10	0.51	50	5.6	0.4
6	0.4	−50	—	—	50	5.6	0.3
7	0.7	−100	—	—	50	5.6	4.0
8	0.8	−400	15	0.22	50	5.6	1.0

Abbreviations: C*, average of subjects with no osteoporosis. Ca intake, average daily intake of calcium in the diet in grams. Ca balance, daily gain or loss of calcium in diet in milligrams per day. $Sr^{85}O$, time in minutes during which cortical bone of the tibia produced an *increase in the counting rate* of the scintillation probe–photomultiplier tube–ratemeter system. A, accretion rate in grams of calcium per day, estimated by method of Bauer *et al.* (1961); Sr^{85} was used in all but Case No. 8, where Ca^{47} was used. Cx, percentage of the thickness of the cortex of the tibia of nonosteoporotic subjects of comparable age. SM, bone weight in kilograms estimated on the basis of normal bone mass = 16 per cent of body weight. TC, total uptake of tetracycline in γ per gram of weight of fresh cortical bone obtained at biopsy.

and phosphorus as patients with 100 per cent of their bone tissue. Tetracycline labeling of osteoporotic bone correlated with chemical balance and radioisotope studies revealed that this activity was accomplished chiefly by the exchangeable, labile, reactive, or metabolic bone tissue—which constitutes only approximately 1 per cent of the total bone mass in normal subjects and perhaps not more than 2 to 3 per cent in osteoporotics. Thus, kinetic studies were unable to provide a measurement of the activity of either the total bone mass or the relatively nonreactive structural bone, which is where the deficiency exists in patients with osteoporosis.

The Progress of Osteoporosis in Five-Year Radiographic Survey of Aged Females

The survey of 100 women with average age of 85, in residence at an Eastern Star Home in Los Angeles, was made in 1957 and reported at the Lankenau Hospital Conference in 1958 (Urist, 1960*b*).

TABLE III. Five-Year Follow-up Radiographic Examinations of 100 White Females, Average Age 85, Previously Reported by Urist (1960*b*)

Radiologic observations	Per cent
Osteoporosis (diagnosis based on collapsed vertebrae and ballooned discs in 1957)	26
Deaths in group of both nonosteoporotics and osteoporotics in period 1957–1962, ages 70 to 103	75
Survivors with osteoporosis, ages 74 to 89	3
Survivors without osteoporosis, ages 71 to 91	22
Further fractures in osteoporotics, 1957–1962	10
Further fractures in nonosteoporotics, 1957–1962	0
Additional cases of osteoporosis appearing in 22 survivors previously free of compression fractures of the spine	0

Table III presents the results of a second radiographic survey designed to determine whether additional cases of the disorder (as diagnosed by collapsed vertebrae) appeared during a 5-year period in previously unafflicted individuals. In addition, there was also the question whether the disorder (also determined by the number of collapsed vertebrae) progressed in proportion to a period of time in the course of aging. Only 25 per cent of the women survived the period between 1957 and 1962; 22 per cent were nonosteoporotics and 3 per cent were osteoporotics. Ten per cent of the osteoporotics, but none of the nonosteoporotics, were treated for further fractures or spontaneous collapses of vertebral bodies during the 5 years. Thus, it appeared that the disorder was progressive in patients who had the disease, but was not incurred by others who had reached the 8th and 9th decades of life. This was clearly evident from the radiographs of 22 women, with an average age of almost 88, who, as yet, had no collapsed vertebrae. It was also evident that osteoporosis generally occurred at a much earlier age, possibly between 50 and

TABLE IV. Indices of Physiologic Aging of Bone and Osteoporosis in White Females between 5th and 7th Decades of Life

Characteristic or component	Estimated per cent change: Aging, <65	Estimated per cent change: Osteoporotic	Authority
Density (w/v, 10 bones)	−12–30	−33–50	Trotter *et al.* (1960); Urist *et al.* (1962)
Bone tissue water	−20	?	Robinson (1960)
Variation in density of osteons	+10	+20	Amprino and Engström (1952); author
No. osteons less than $\frac{3}{4}$ closed	+20	+30	Jowsey (1960)
Enlarged vascular channels, porosity	+10	+20	Jowsey (1960)
Vascular channels with plugs of amorphous calcium deposits	+2	+5	Author
Osteocytolysis	+15	+30	Sherman and Selakovitch (1957); Casuccio (1962); author
Osteocyte lacunae filled with calcium salts	+1	+5	Casuccio (1962)
Surface area occupied with resorption	+10	+15–40	Jowsey (1960); Frost (1961)
Rate of retention of bone-seeking isotopes	−10	−15	Bauer *et al.* (1961)
Hexosamine/collagen ratio	−10	−20	Sobel *et al.* (1954); author
EDTA-soluble fraction of mucopolysaccharides	−20	−50	Casuccio (1962)
Degraded acid-insoluble collagen (solid residue)	±1	+10	Little *et al.* (1962)
Loss of fibrillar form of architecture of collagen	±1	+10	Little *et al.* (1962)
Bone highly reactive with tetracycline	+10	+10	Author

65, and did not progress at an increasing rate with advancing time. The indices listed in Tables IV and V indicate that all morphological and chemical changes attributed to aging occurred in osteoporotics, probably at an earlier age and to a more extreme degree. These observations were interpreted to suggest that osteoporosis is caused by an aberration or acute development of the aging changes in bone, perhaps in persons who are genetically predisposed to rapid earlier deterioration of the skeletal system.

TABLE V. URINARY INDICES OF PHYSIOLOGIC AGING OF BONE AND OSTEOPOROSIS

Characteristic or component	Estimated per cent change: Aging non-osteoporotic	Estimated per cent change: Osteoporotic	Authority
Response to ACTH	±10	±15	Urist (1960*b*); Gallagher and Spencer (1962)
11-deoxy-17-ketosteroids	−5	−10	Urist and Vincent (1960)
Calciuria	0	+20	Urist (1962)
Ability to reduce total urinary calcium on low calcium diet	+10	−25	Nordin (1962*c*)
Calcium excretion index, Ca^{45}/Ca^{40}, accumulative	+5	+10	Bronner *et al.* (1962)
Free hydroxyproline	+10	+20	Raveni *et al.* (1962)
Mucopolysaccharide	+10	+20	Bertolin and Greco (1962)

ACCELERATED CHANGES OF AGING IN OSTEOPOROTICS

The distinguishing feature of patients with severe osteoporosis, described in Table II, as compared with patients of comparable age without osteoporosis, was *bone failure* or collapse of the vertebral bodies of dorsal and lumbar spine. Figure 12 illustrates the relation between the reduction in bone mass and the age. The number of osteoporotic patients was too small for statistical evaluation, but sufficient to suggest a relationship to aging. The shape of the curve that describes the rapid rate of reduction of bone mass with time in osteoporotics, and the more gradual decline that describes the rate of normal or physiologic aging, indicated that the disease is most active during the period from 50 to 65, and may progress, but rarely arises, after age 75. The various tissue (Table IV) and urinary (Table V) indices of change in bone structure and metabolism that have been estimated and attributed to physiologic aging were also found in patients with osteoporosis. The large amount of tissue with empty lacunae and the patches of necrotic bone suggested bone circulatory insufficiency in osteoporotics to a greater degree than in nonosteoporotic aged persons (Table VI). Spontaneous fracture or bone failure occurs in the spine, the ribs, and the neck of the femur, where the cortex is thin and the necrotic osteoporotic bone is revas-

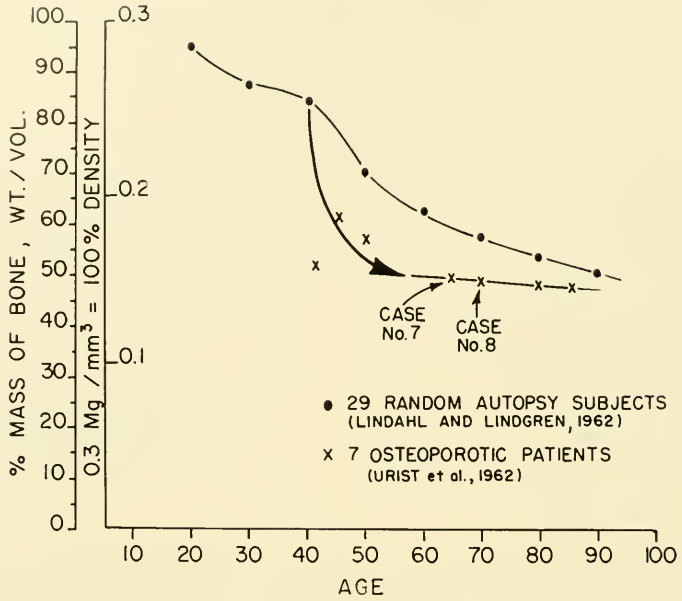

FIG. 12. Graph illustrating relation between change in bone density and age in random autopsy subjects and in patients with severe osteoporosis. The amount of retention and the rate of change in bone mass after age 70 is almost the same in the two groups of white women. The important characteristic of the group with severe osteoporosis is the sudden change in the quantity of bone retained in the skeleton between 50 and 65 years of age. The chief feature distinguishing the osteoporotic group from the group with physiologic rate of decrease in bone mass or aging was bone failure, or spontaneous collapse of vertebral bodies. It was assumed that borderline cases of osteoporosis, with or without slight bone failure, could occur in older age groups, but the separation of the two groups was generally quite clear-cut.

cularized and resorbed too fast to be replaced by new bone (Figs. 13A to 13C).

RECENT AND RELATED LITERATURE

Race, Sex, and Genetic Factors

Moon and Urist (1962) reported 87 cases, 77 women and 10 men, with severe osteoporosis admitted to a new hospital that received 90,000 patients over a period of 4 years. All were Caucasian. Trotter *et al.* (1960) measured the density of whole bones of anatomical specimens by the ratio of weight to displacement volume and ob-

TABLE VI. Osteocyte Counts: Number of Stellate (S), Retracting (R), Calcified (C), and Empty (E) Lacunae per High Field in Cortical Bone in Osteoporotic and Nonosteoporotic Autopsy Subjects

		Osteocytes											
		Inner lamellae				Outer lamellae				Interstitial lamellae			
Case no.	Age	S	R	C	E	S	R	C	E	S	R	C	E
1	42	3	3	1	0	1	6	3	1	0	5	0	24
2	44	3	2	1	1	1	5	4	3	0	4	1	26
3	50	3	4	0	0	1	6	3	3	0	5	0	30
4	54	3	3	1	0	2	6	3	4	0	8	0	29
5	76	3	2	1	0	1	6	5	3	1	4	0	32
6	84	3	2	1	1	1	6	3	3	0	7	1	28
7	67	2	3	2	1	0	5	4	4	0	5	2	30
8	71	2	2	3	1	1	7	5	4	0	7	0	27
Average osteoporotic	63	2.7	2.6	1.2	0.4	1.0	6.0	3.7	3.0	0.1	5.7	0.5	28.2
C1	48	7	1	0	0	4	5	1	1	8	6	0	15
C2	50	7	1	0	0	4	4	0	1	8	6	1	18
C3	71	7	1	1	0	4	2	2	2	4	3	2	27
C4	80	5	2	1	1	3	4	3	2	1	2	1	28
Average nonosteoporotic control	62	6.5	1.2	0.5	0.2	3.7	3.7	1.5	1.5	5.2	4.2	1.0	22.0

served that the bones of white males were normally denser than those of females; Negro females had denser bones than white; the rate of decline with age was parallel in all sex and age groups. Smith *et al.* (1960) made an x-ray survey of 218 ambulatory outpatient cases with osteoporosis; almost all were Caucasians, a very few were of Latin groups, and none were Negro, although the hospital was geographically located in the area of a large Negro population. This observation was reinforced further by the fact that the incidence of spontaneous fracture of the hip, a common symptom of osteoporosis in patients over 70 years of age, was very much lower in Negro than in white racial groups (Moldawer *et al.*, 1961). Vincent and Urist (1961) reported that osteoporosis occurred in a high

FIG. 13A. Radiograph showing the head and neck of the femur (natural size) excised following a bilateral intertrochanteric fracture in a 78-year-old woman with severe osteoporosis. The split in the superior margin of the head was produced by insertion of a hip skid. Note the accentuation of the vertical trabecular markings. The thin cortex and hollow neck are indications for excisional surgery or bone pegs, rather than internal fixation with metallic appliances.

FIG. 13B. Microradiograph of the superior cortex of the neck of the femur in the case shown in Fig. 13A. Note the hypermineralized avascular bone on the right and the attenuated discontinuous cortex in the center. (× approx. 75.)

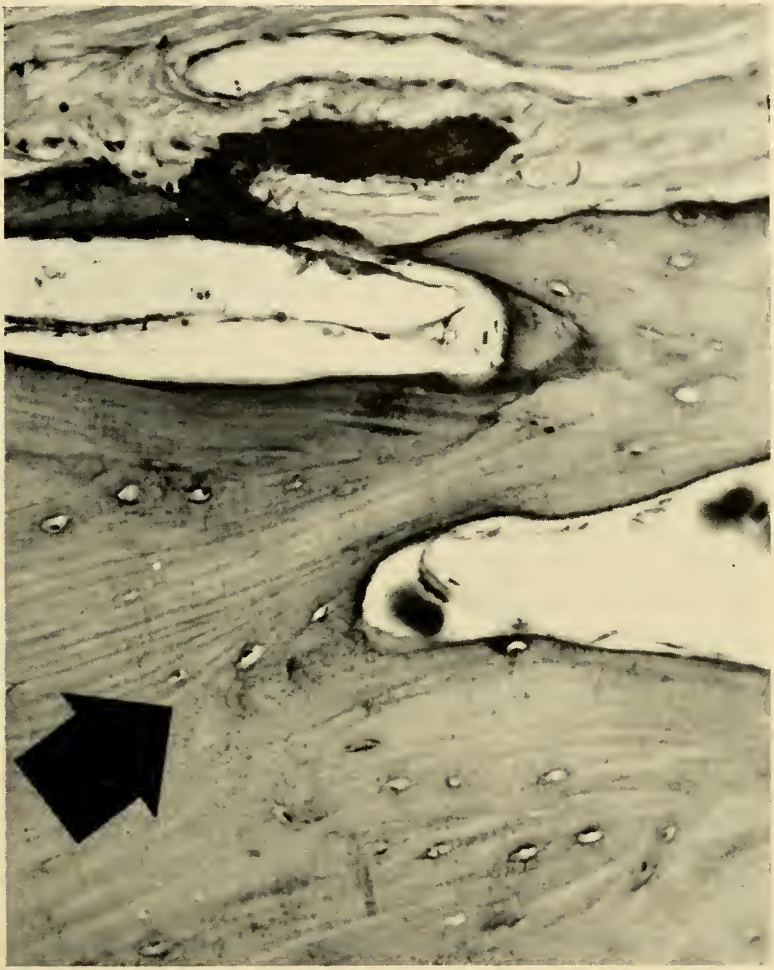

FIG. 13C. Photomicrograph of the center of the cortex of the neck of the femur shown in Figs. 13A and 13B. The visceral capsule or vestigial periosteum of the hip is shown at the top. The dense-staining bone matrix lies beneath it. The lacunae are either empty or partly filled with pyknotic nuclei and abnormal osteocytes (arrow). (× approx. 400.)

percentage of males with chronic debilitating diseases, but the condition was invariably less severe than in females. The possibility of a hereditary or genetic factor associated with osteoporosis of the

spine has long been suspected but has yet to be subjected to a systematic investigation.

Densitometric Methods of Diagnosis

Renewed effort has been made to perfect densitometry to enable physicians to diagnose osteoporosis at an early stage of the disease and evaluate the efficacy of different methods of treatment. Nordin *et al.* (1962*a*) devised an interesting technic in which lateral tomograms were prepared of the patient and a dissected specimen of the lumbar spine was placed next to the subject. The film images were fed through a Laurence-Locarte recording densitometer. The ratio of the densities of the subject and the standard was called the *relative vertebral density.* It was concluded that the codfish spine occurred when the vertebral body was less dense than the intervertebral discs. Mayo (1961) made a similar survey and concluded that the individual could lose a relatively large amount of bone mineral and still fall within the range of normal individuals, and that the early diagnosis of osteoporosis is limited by these considerations. Such limitations did not apply to the use of quantitative methods for measuring changes in the bones with time in the same individual. Doyle (1961) surveyed the proximal end of the ulna, and Wagner and Schaaf (1961) examined the thumb, and corroborated these results.

Vose (1962) described microradiographic changes in cortical bone. The mineral content varied from 0.94 to 1.19 gm of hydroxyapatite per cc of bone in normals and from 1.12 to 1.50 in osteoporotics. Thus, while the bone mass was reduced in volume in osteoporosis, the bone tissue that persisted was hypermineralized. This increment was apparently in amorphous plugs in blood vessels and in the interstitial lamellae of old deposits. In view of the technical difficulties, physical variables, and other complexities of conventional x-rays for measurement of bone density, it is now desirable to search for entirely different principles for obtaining quantitative information about osteoporosis. Jacobson (1962) is testing dichromatic absorption radiography for calcium in mineralized tissues; this method gives greater contrast than an ordinary radiograph (Jacobson, 1953).

Physiologic Aging of Bone

Structural physical and chemical alterations occur in the human skeleton in the course of chronological or physiologic aging. Sherman and Selakovitch (1957) noted that osteocytes begin to disappear from the outer lamellae of haversian bone during adolescence, and the number of empty lacunae increases after the 3rd decade. These changes were attributed to circulatory insufficiency and physiologic necrosis of bone in old age. Atkinson *et al.* (1962) measured the dry weight and displacement volume of blocks of bone, $w/v = D$, and described decrease in thickness and density of cortical bone with increasing age in both sexes after 50; the metaphyseal decreased faster than the diaphyseal cortex. Sissons (1962) noted that in the aged many osteons ceased to grow when they were half closed, and confirmed Amprino and Engström's (1952) observation that the time between osteon formation and completion of mineralization increased with aging. In aged individuals the osteon became rapidly mineralized to 90 per cent of the capacity, but a larger proportion of the total bone was always less than 100 per cent mineralized. It was easy to distinguish the incompletely mineralized old bone from new bone because the osteon in which old bone is found contains a sclerotic inner edge before it is half closed.

Lindahl and Lindgren (1962) observed that in 60 subjects between 14 and 91 years of age, the decrease in bone density was parallel in the upper end of the tibia and the vertebral column. Samples of bone were treated with hot water to remove marrow and with xylene to remove fat, and cut into cubes to measure volume of bone tissue; the density of the sample was the weight per unit volume of the cube. Irrespective of age, osteoporosis was present when the density was 0.20 mg per mm^3 of spongy bone. These observations confirmed the conclusions of Trotter *et al.* (1960) that bone density diminishes with age. This is a generalized process, but the rate differs in different bones, in males and females, and in the white and Negro races. According to Morgan *et al.* (1962), the decline was more pronounced between ages 50 and 65 in women, and usually did not occur in men until age 70. In women, the

severity of the radiographic changes had no relation to the length of time following the menopause (Moon and Urist, 1962).

The relation between the time-dependent process of aging and osteoporosis was obscure. Groen *et al.* (1960) described alveolar atrophy in osteoporotic subjects and concluded that senility was not the cause. Berglund and Lindquist (1960) described osteoporosis or osteopenia in adolescents. The appearance of the disorder in rare instances in young men, the so-called idiopathic osteoporosis (Hall and Kennedy, 1962), also suggested that a specific factor, in addition to physiologic aging, endocrinopathy, and nutrition, had to be taken into consideration.

Degradation of the Collagen of Bone

An interesting theory appeared in 1962 to propose that osteoporosis is primarily a degenerative disease, rather than an endocrine or metabolic disorder. Little *et al.* (1962) examined normal and osteoporotic bone tissue by electron microscopy and x-ray diffraction of the fibrous proteins. Bone was defatted with alkali and decalcified with citric acid in order to enhance the solubility of collagen in acid buffer, and, thereby, separate soluble eucollagen. This was reprecipitated in pure form by dialysis. The nonsoluble collagen and ground substance were analyzed together as solid residue. The x-ray diffraction pattern of the solid residue suggested that the crystal structure of the collagen was altered or degraded. Since these alterations were not seen in bone from nonosteoporotic aged individuals, or in dead bone, and resembled degradation produced by irradiation damage, it was suggested that an active degrading agent may be involved. The patterns also included the lines of a fatty acid, possibly myristic acid. Electron micrographs of bone showed loss of the normal architecture and fibrillar form of the collagen in various areas of osteoporotic bone. Little *et al.* (1962) suggested that osteoporosis was the result of a local agent emanating from osteocytes rather than a general chemical effect. The composition of the mineral phase of bone was the same in osteoporotic and nonosteoporotic individuals of comparable age, and the apatite crystallites probably played only a passive role (Holdeway *et al.*, 1962). The degradation of collagen by irradiation and starvation,

and the inhibition of conversion of soluble to insoluble collagen in irradiated experimental animals (Gerber *et al.*, 1962), illustrated some of the possible derangements that could occur in aging and osteoporotic bone.

The level of the urinary hydroxyproline has also been interpreted to reflect either the process of degradation or turnover of bone collagen. Bastomsky and Dull (1962) described hydroxyprolinuria in rats treated with parathyroid hormone and triiodothyronine. Klein *et al.* (1962) found a 60 per cent increase in a patient with hyperparathyroidism with bone disease, as compared with no increase in two patients without bone disease. Raveni *et al.* (1962) measured total and free urinary hydroxyproline in normal and osteoporotic patients and concluded that an increase in the free fraction reflects increased destruction or catabolism of bone in this disorder. Ibsen *et al.* (1963) evaluated a proline tolerance in rabbits with healing fractures and concluded that bone formation increased the rate of proline utilization. Further investigations may establish proline metabolism as a useful indicator of bone matrix metabolism in health and disease.

Alterations in the Mucoproteins of Bone

Casuccio *et al.* (1962) described abnormalities of the osteocytes in bone tissue and a decrease in several fractions of the mucopolysaccharides of bone matrix in the skeleton of aged and osteoporotic subjects. The perilacunar sheath of Rouget-Neumann was accentuated, metachromasia was absent, nuclei were pyknotic, the canaliculi were reduced in number and showed terminal club-shaped thickenings. The osteocyte sheaths were shrunken, shriveled, or absent. The mucoprotein cylinders that normally fill out the canaliculi were absorbed and the osteocytes retracted their cytoplasmic processes. This suggests intralacunar resorption or osteocytolysis as described by Heller *et al.* (1950). Casuccio (1962) observed a 50 per cent decrease in protein nitrogen but only a 6 to 9 per cent decrease in inorganic mineral in bone tissue in osteoporotic patients; the bones of aged individuals without osteoporosis were similarly altered, although to a lesser degree. Four fractions of the mucopolysaccharides of bone were influenced by both aging and osteoporosis

as follows: A versene-soluble component, rich in galactosamine, was reduced 50 per cent, and the galactosamine/glucosamine ratio was changed from 3:1 in young to 1:8.1 in aged or osteoporotic subjects; a versene-insoluble component, consisting of collagen and interfibrillary substance, was altered so that the hydroxyproline was relatively high and the amino sugar content was relatively low; an alkali-soluble component, consisting of collagen, was unremarkable; an alkali-insoluble fraction, consisting of a small amount of uncharacterized residue rich in carbohydrate, did not change with age. These observations suggested that the interfibrillary mucopolysaccharide gradually decreased progressively with aging and more abruptly with osteoporosis of bone. Bertolin and Greco (1962) measured an increased daily rate of excretion of a mucopolysaccharide with a high galactosamine/glucosamine ratio in the urine of patients with osteogenesis imperfecta and osteoporosis. These observations substantiated the findings of Sobel *et al.* (1954) and Sobel (1960) that the rate of deposition of hexosamine-containing substances decreases in the skin and bones of rats with the progress of growth and aging. These changes were probably unspecific, but it would be of interest to know whether they occur in osteoporosis associated with endogenous and exogenous hyperadrenal corticoidism, hyperthyroidism, acromegaly, and other endocrinopathies.

Nutrition and Metabolism of Calcium

Though a calcium-deficiency disease has never been clearly established as an entity in adult human beings (Nicolaysen, 1960), it is generally assumed that low calcium intake is *one* of the factors in the development of osteoporosis (Malm, 1961). The hypothesis rests on the observation that some patients who are in negative calcium balance on 0.8 gm per day will retain substantial amounts on an intake of 2.0 gm. Nordin (1962*a*) reasoned that if osteoporosis is due to increased bone resorption, one would expect to find reduced calcium intake, malabsorption, or high excretion in osteoporosis. A dietary survey revealed that 128 patients with osteoporosis had a significantly lower intake of calcium and protein than 128 patients with normal bones (Nordin, 1962*b*). As many as 15 per cent had steatorrhea, but this disorder did not impair the ab-

sorption of calcium when the intake was raised to 2.0 gm per day. Some patients with osteoporosis had pyelonephritis and a "calcium leak" that caused negative calcium balance (Nordin, 1962*b*). Of 29 balance studies on 17 patients, 5/17 were in negative calcium balance, and 12/17 were in slightly positive calcium balance. Inasmuch as the calcium lost in sweat was not measured in balance studies, it was assumed that all the patients were in negative calcium balance. The contention was, first, that osteoporotics were not able to reduce their urinary calcium on low calcium diets, but had the ability to retain calcium on high calcium diet (Bhandarkar and Nordin, 1962); second, that the cause of the disorder was prolonged negative calcium balance in subjects with higher than normal calcium requirement or inability to adapt to inadequate intake. Some severe osteoporotics, however, were able to adapt to low calcium intake. In any case, the condition was relieved by dietary supplements of calcium, but x-ray was not sensitive enough to detect the new bone that was presumed to have been added (Nordin, 1962*c*). Bhandarkar *et al.* (1961) fed 7.5 μc of Ca^{47} or 5 μc of Ca^{45} to measure calcium absorption, and noted that the majority of patients with osteoporosis absorbed calcium normally. Kinetic studies were interpreted to show that the rate of bone accretion in osteoporotics was also the same as normal (Nordin *et al.*, 1962*b*).

Fraser (1962) divided patients with osteoporosis into three groups, Cushing's syndrome, atypical or idiopathic, and typical or postmenopausal, and corroborated Nordin's contentions. However, sex hormones were prescribed in addition to calcium supplements for senile osteoporosis. Haas *et al.* (1962) measured retention of an infusion of calcium in 36 osteoporotics and 12 normal subjects, and concluded that the lowest levels occurred in Cushing's syndrome, castration, and idiopathic osteoporosis.

In the past, some investigators have assumed that typical osteoporosis represents chronic low-grade hyperparathyroidism of bone. The calcium deficiency hypothesis ultimately leads to such an assumption for an explanation of the bone tissue deficit. Gordan *et al.* (1962) rejected hyperparathyroidism secondary to malabsorption of calcium as a cause of osteoporosis; patients with hyperparathyroidism had only 35 to 75 per cent tubular reabsorption of phos-

phate, whereas patients with osteoporosis had 80 to 100 per cent. Goldsmith *et al.* (1962) employed an infusion of 180 mg of calcium in the form of calcium glucoheptonate and observed that phosphate excretion was unchanged in hyperparathyroidism, but lowered in other bone conditions. Frost (1961) measured the number of Howship's lacunae per unit volume of bone in undecalcified thin ground sections of cortical biopsies of 12 cases of osteoporosis, and concluded that osteoclasis was four times greater than in normal subjects. Though bone biopsies and tests of intestinal tract and kidney excretion of calcium and phosphate were extremely valuable, it was not possible to diagnose low-grade hyperparathyroidism without an exploration of the neck and a positive identification of enlarged parathyroid glands (Henneman *et al.*, 1958).

Adrenal Cortical Hormones and Intermediary Metabolism of Protein

Postmenopausal or senile osteoporosis is radiographically and histologically indistinguishable from osteoporosis associated with Cushing's syndrome, either the endogenous or the exogenous form (Murray, 1961). The mechanism of depletion of the bone tissue by the action of glucocorticoid hormones has been attributed to inhibition of formation of osteoblasts with relatively little effect on the normal bone-resorbing activity of osteoclasts. Suppression of bone formation alone explains the decrease in absorption of calcium from the intestinal tract that is generally found in Cushing's syndrome, but direct effects have also been reported by Bhandarkar *et al.* (1961). Children and women after the menopause were more susceptible than men to bone destruction by cortisone (Schlesinger *et al.*, 1961). Anabolic steroids protected, but vitamin D increased, the vulnerability of the skeleton (Bassett *et al.*, 1960).

One of the intriguing aspects of the mineral metabolism was the effect of adrenalectomy. Patients with endogenous Cushing's syndrome were relieved of a backache and further fractures of the spine, yet the radiographic picture was relatively unchanged following this operation (Martinez and Greenblatt, 1962). Myers (1962) reviewed the literature on calcium metabolism in adrenalectomized animals, including patients with Addison's disease, and concluded

that the adrenal cortex has a dual effect upon calcium metabolism. Excess glucocorticoids induced negative calcium balance, and insufficiency caused change in calcium equilibrium characterized by hypercalcemia. The latter was independent of the bone, and the mechanism was different from that produced by parathyroid hormone. Furthermore, this hypercalcemia persisted in parathyroidectomized animals, and cortisone in physiologic amounts restored the serum calcium to normal (Kolb and Cohen, 1962). Walser *et al.* (1962) described 58 samples of the serum of adrenalectomized dogs and attributed the hypercalcemia to a combination of factors, such as hyperproteinemia, due to hemoconcentration; increased affinity of calcium for protein, due to hyponatremia; increased citrate levels, as much as three times normal; and increased amounts of unidentified calcium complexes. The effective or free calcium ion concentration was not altered by adrenalectomy.

Urist (1960*b*) reported the case of a 78-year-old woman with severe osteoporosis treated by adrenalectomy. The glands were low in weight, as is usual in persons of advanced age, and there was atrophy of the zona fasciculata of the right, but not of the left, adrenal cortex. Urist and Vincent (1960) observed that the decrease in urinary 11-deoxy-17-ketosteroids that occurs in aged women was more extreme in women with severe osteoporosis. Caldwell (1962*b*) examined the adrenal glands in 31 autopsy subjects, including 8 cases of senile osteoporosis, and found no evidence of involution or atrophy, and possibly slightly greater than normal adrenal cortical cell activity. These observations suggested that further observations upon the adrenal cortical hormone metabolism in castrates and aged patients with osteoporosis may be of considerable value. For some unknown reason, adrenalectomy increased the sensitivity of animals to the side effects of glucocorticoid hormones. Both the adrenals and the gonads are markedly diminished in size in birds with osteoporosis (Urist, 1958). Whereas the influence of adrenal hypercorticoidism upon osteoporosis was clearly demonstrable, the effect of endocrine deficiency disorders was quite obscure, and an antiosteoporosis factor produced by the adrenal cortex, the liver, or the skeleton itself, but dependent upon normal endocrine gland function, had to be considered (Urist, 1960*b*).

Teng *et al.* (1961*a*, 1961*b*) described osteoporosis in 7 children with a systemic disorder of protein metabolism caused by tumors, cirrhosis, and von Gierke's disease. As in patients with senile osteoporosis, the urinary excretion of calcium was decreased, owing to the low rate of turnover of bone tissue. Apparently skin, muscle, and bone had a low priority rating and were deprived of their supply of proteins to fulfill the needs of organs of vital bodily functions. A 60-year-old osteoporotic woman, who had only 50 per cent of the normal bone mass, was calculated to have had a 10 per cent deficit in body protein. Adrenal 11-oxycorticosteroids decreased the rate of protein synthesis, or increased the rate of protein breakdown. Hence, metabolic reactions, nutritional factors, and hormonal influences were interdependent, and the rate of differentiation of osteoblasts and deposition of bone must have been regulated by each and all.

Skeletal Kinetics

The recent literature on measurement of turnover of bone tissue by tracer techniques with radioisotopes, stable strontium, and tetracycline has already been reviewed in detail (Bauer *et al.*, 1961; Eisenberg and Gordan, 1961; Urist, 1962; Urist *et al.*, 1962). When the results of two or three tracer technics were correlated with the results of conventional chemical balance studies and bone biopsy, we concluded that the use of tracer dilution formulas measured the activity of less than 1 per cent of the total bone tissue (Urist *et al.*, 1962) and gave no information about the volume of the total bone mass. Less than 1 per cent of the skeleton was capable of turning over 0.5 to 1.0 gm of calcium, the daily requirement of an adult human being. Therefore, the defect in osteoporosis was in the process of storage of calcium in structural bone, not in the activity of reactive or metabolic bone. Nevertheless, Eisenberg and Gordan (1961) identified two divergent groups in 26 patients with osteoporosis. One group had small miscible pools and low bone formation rates, as, for example, osteoporotics of advanced age or with Cushing's syndrome. Another group had large miscible pools and rapid bone formation rates, as, for example, in thyrotoxicosis and acromegaly, in which there is excessive bone resorption. Thus, in cases of

extreme alteration in the structure of the bone tissue, the percentage of the skeleton that was reactive or exchangeable with tracer substances was proportional to the volume of the percentage of skeleton that was relatively nonreactive or nonexchangeable. Our impression was that in individuals in early stages of the disease, in which quantitative data would have been most valuable for diagnosis and treatment, it was not possible to distinguish normal from osteoporotic individuals. Further development of the mathematical treatment of radioisotope kinetic data and the osteogram of MacDonald (1960) may increase the value of measurements of skeletal turnover with Sr^{85}, Ca^{47}, and other isotopes in patients with local, as well as systemic, bone disease (Dow and Stanbury, 1960; Greenberg *et al.*, 1961; Bauer *et al.*, 1961).

Fractures

We assumed in previous reports that physiologic aging or atrophy of the spine did not produce gross deformities or spontaneous collapse of vertebrae, and that radiographic evidence of multiple compression fractures, ballooned discs, and thin cortex distinguished osteoporosis as a disorder (Urist *et al.*, 1959; Urist, 1960*b;* Urist and Moon, 1961). The degenerative changes in the bone tissue were the same in many respects in physiologic aging and in osteoporosis (Table V), and the only difference could have been in the rate of decline of the bone mass. In both conditions, the ratio of weight to volume or density declined from 0.3 to 0.2, but in osteoporosis the decline occurred at an earlier age and a faster rate (Fig. 12). *Bone failure* or spontaneous fractures of the spine occurred when the density fell below 0.15, or 50 per cent of that of the normal adult human skeleton at age 20. It was interesting in this connection that many patients with osteoporosis reported sudden and recent weight loss.

The high incidence of fractures in the aged has been attributed to lower bone density, but the correlation with osteoporosis is incomplete. The incidence of severe osteoporosis is 26 per cent in healthy aged women (with average age 85), but it is 76 per cent in women, average age 73, with all types of fractures of the hip (Urist *et al.*, 1959; Urist, 1960*b;* Urist and Moon, 1961). Stevens

et al. (1962) noted that fractures of the hip are not more common in osteoporotics until after 70 years of age, but that intertrochanteric fractures are more typical of osteoporosis than other types. Although the aged are more susceptible to fractures because of the physiologic decline in bone density, it is clear that incidence of fracture increases further in individuals in older age groups with severe osteoporosis. Previously, Buhr and Cooke (1959) attributed fractures of the hip to "senile matrix of old age." Little *et al.* (1962), however, contended that the bone matrix was degraded or defective in severe osteoporotics, as compared with nonosteoporotic individuals of comparable age.

Osteoporosis in Experimental Animals

The discovery of osteoporosis in the White Leghorn hen (Urist and Deutsch, 1960*a*) was followed by the realization that the loss of bone mass is a manifestation of cage layer fatigue (CLF) (Urist, 1960*a*). Urist (1959) had previously observed that hypertrophy of the adrenal cortex developed when normal White Leghorns were fed a calcium-deficient diet. But the same variety of laying hens with CLF had atrophied adrenal glands, and the relationship of adrenal hypercorticoidism to avian osteoporosis has not been clarified. The problem is the same as with osteoporosis in man. Severe osteoporosis was produced in hens by administration of ACTH (Urist and Deutsch, 1960*b*) or cortisone (Urist and Deutsch, 1960*c*). Roosters were highly resistant; caponized birds were susceptible to bone-destructive properties of these agents. The skeleton of the rooster initially possessed a larger mass of bone than that of the capon or hen, and required a longer period and a larger amount of bone resorption to develop the disorder, but other factors may have been involved. It was not possible to deplete the skeleton of the rooster to the extent that occurs in the hen with CLF.

Taylor *et al.* (1962), Taylor (1962), and Bell and Siller (1962) corroborated our observation that osteoporosis is the basic lesion in CLF, and proposed that the cause was genetic and the mechanism was hyperactivity of the pituitary. Bell and Siller (1962) described 40 cases in Brown Leghorn hens, and noted hyperosteoclasis of cortical bone and atrophy of muscle as well as bone. Birds with

CLF fed a low-calcium diet continued to lay heavily and produced well calcified eggshells. Normal birds produced one or two thin-shelled eggs and discontinued ovulation when the intake of calcium was restricted. Taylor *et al.* (1962) suggested that the pituitary cut-off mechanism was generally very efficient, but lines bred for intensive laying continued to ovulate when transferred to a low calcium intake, and it was these individuals that were susceptible to CLF. Restriction in cages and insufficient physical activity was an important factor in CLF; some birds recovered simply by being released to exercise ad libitum. Thus, in birds as in humans, calcium-deficient diet and sedentary life were precipitating factors in the pathogenesis of osteoporosis, but the main cause was unknown, although probably genetic in origin.

Silberberg and Silberberg (1962) reported that senile osteoporosis in mice is associated with genetic factors. The DBA strain was more susceptible than other inbred strains to a disorder characterized by thin, rarefied, deformed bones appearing between 24 and 36 months, an advanced age for mice. Young rats fed diets with less than 0.1 per cent calcium and some vitamin D develop osteoporosis that is cured by treatment on high calcium intake (McClendon *et al.*, 1962). During adult life, rats retain their bone mass more tenaciously than other mammals, and are generally resistant to osteoporosis (Nicolaysen, 1960). Despite continuous administration of cortisone, in doses that produce osteoporosis in birds, mice, rabbits, and man, rats will retain the bulk of the calcium in the skeleton. Caldwell (1962*a*) noted that cortisone inhibits bone growth, but the effect in rats is temporary, and these animals develop resistance to the hormone. Furthermore, by measuring the amount of calcium per gram of wet weight of whole bones, Caldwell (1962*a*) observed that adrenalectomy and orchiectomy destroyed the resistance to cortisone, and high calcium intake did not prevent or cure the osteoporosis. Urist, MacDonald, Johnson, Deutsch, and Vincent (unpublished) performed similar experiments and measured the capacity of the adreno-orchiectomized rat to retain an injection of Sr^{85}, but found no significant abnormality of bone. Nevertheless, adrenalectomized animals are more generally sensitive than normal animals to cortisone, and further experiments on osteoporosis in

cortisone-treated adrenalectomized rats would be of great interest. The possibility of an antiosteoporosis factor present in normal adult males, and essential for bone retention during aging, requires intensive investigation (Urist, 1960*b*, 1962).

The action of adrenal cortical hormone upon bone in rats was attributed to inhibition of absorption of calcium from the intestinal tract or physiologic calcium deficiency. Malabsorption was assumed to stimulate the parathyroids and lead to osteoporosis. Clark and Smith (1962), however, noted hypercalciuria in parathyroidectomized rats, the same as in normal rats, and Clark and Roth (1961) concluded that hydrocortisone decreased the reabsorption of calcium and phosphate by the kidney tubules. Under these conditions, weanling rats increased the density of their bones, but grew at a rate so much less than normal that presumably the over-all retention of calcium was less. The reaction of the skeleton has long been known to be entirely different before and after a rat reaches maturity and the plateau of growth, and the endocrine relations were different in many respects in rats, other rodents, and higher mammals. Calcium deficiency was a contributing factor in the development of osteoporosis; Storey (1961) fed low-calcium diets to rabbits treated with cortisone and described osteoporosis associated with extensive osteoclastic activity and secondary hyperparathyroidism.

How endocrine interrelationships influence osteoporosis in man is not known. Schlesinger *et al.* (1961) observed that adrenal steroids produce osteoporosis in children and postmenopausal women more frequently than in men, and generally from treatment on lower doses and in less time. It could have been argued that the reason is simply that young adults have more bone to begin with than children and senile women. However, the subject was considerably more complex; 85 per cent of the patients of comparable ages and skeletal conditions did not develop osteoporosis on comparable steroid therapy. Other, as yet unknown, factors were evidently of primary importance.

Kao *et al.* (1962) reported that the glycoprotein decreased in bone and increased in costal cartilage in rats in the process of aging between 1 and 36 months. Previously, Sobel *et al.* (1954), Sobel and

Marmorston (1958), and Sobel (1960) observed that the hexosamine/collagen ratio decreased in the femora of rats with aging. In view of recent reports of decrease in galactosamine-rich mucoproteins of bone matrix of patients with osteoporosis (Casuccio, 1962), it is now necessary to assume that qualitative, as well as quantitative, changes occur in the skeleton in aged and osteoporotic bones. The nature of the relation between the time-dependent process of aging and osteoporosis has not been defined in meaningful terms.

Treatment

A record of the progress of a control series of cases of osteoporosis over a long period of time is essential for evaluation of any one, or any combination, of the many forms of treatment that have been prescribed for the disorder. It was possible that in many patients the skeleton was reduced within a few years to 70 or 50 per cent of normal bone mass, and there was no further progress of the disorder over a period of 5 to 20 years. This possibility created uncertainty about whether therapy had had even a preventive effect either on further loss of bone or on the occurrence of spontaneous fractures (Urist, 1962). Though sex hormones (Gordan, 1961; Vincent and Urist, 1961), anabolic steroids (Kuzell *et al.*, 1962), fluoride (Rich and Ensinck, 1961), calcium supplements (Nordin, 1962*a*, 1962*b*), or combinations of these agents (Lichtowitz *et al.*, 1962; Riccitelli, 1962) produced symptomatic improvement, it was reasonable to assume that placebo effects were responsible in many cases. By means of Ca^{45} kinetic studies, Bhandarkar *et al.* (1961) claimed to produce an increase in rate of bone formation from calcium supplements, and Bronner *et al.* (1962) measured acceleration of rate of bone uptake following estrogen therapy. Improvement in radiographic density or increase in the mass of bone tissue in biopsies in series of cases of osteoporosis is essential for proof of a curative effect, but this has not been accomplished (Fig. 14).

Discussion

Present knowledge of osteoporosis consists of a collection of a large quantity of negative and a small quantity of positive informa-

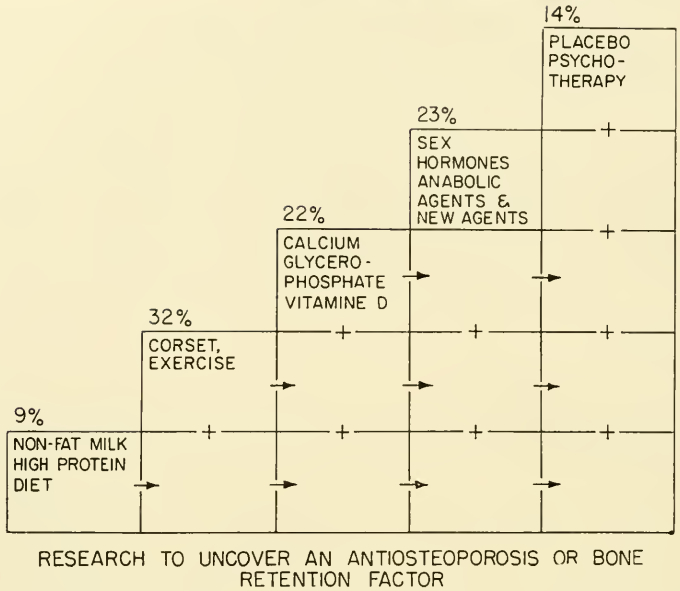

FIG. 14. Diagrammatic illustration of the treatment recorded in the hospital charts of 100 patients with severe osteoporosis over a period of 10 years. Nine per cent changed their diet to include a higher intake of dairy food and protein; 32 per cent made some effort, in addition to the alterations in diet, to increase the amount of physical exercise, and also to wear a corset for comfort; 22 per cent, especially those intolerant of dairy food, received calcium supplements, chiefly calcium glycerophosphate, and maintenance doses (1000 IU) of vitamin D; 23 per cent received sex hormones or anabolic steroids in addition to the above measures; 14 per cent received tranquilizers, muscle relaxants, vitamin B, and other drugs that had placebo effects in that there was no detectable influence upon the skeletal system. No improvement in bone density was observed by serial radiographs of the spine following any one, or any combination, of these treatments.

tion. The etiology of osteoporosis is not known. The disorder appears in its severest form much more frequently in females than in males. Sedentary life after the menopause seems to be a factor. Men and women who do heavy labor usually have spinal osteophytes (spondylosis) and high bone density, whereas sedentary individuals with osteoporosis generally are free of spondylosis and have low bone density. Many patients with this disorder have the normal capacity to produce new bone, but by some insidious mechanism all lose a large percentage of their bone mass in a relatively

short time, perhaps one or two years, generally at some interval between ages 50 and 65. Recent writers contend that polysaccharide is lost, that collagen is degraded, and that the disorder is basically a degenerative process. The morphology and biochemistry of the changes in bone associated with osteoporosis resemble in most respects abnormalities attributable to the physiologic process of aging. The disappearance of osteocytes and the occurrence of patches of necrotic bone are striking.

It appears that in the aged, and more so in the osteoporotic, the movement of calcium from the gut to the blood plasma is slower than normal, movement of calcium from bone to extracellular fluid is accelerated, and movement of calcium from plasma to feces and urine is normal or high. Emphasis is often placed upon the low calcium intake and insufficient peptides (from protein) in the diet in osteoporotic women. Our view is that negative calcium and nitrogen balance may reflect the process of resorption of dead bone and may be the result rather than the cause of osteoporosis. Bone mineral is among the products of breakdown of structural bone, and this is the source of the excess calcium excreted in both the urine and the feces. A low level of osteogenetic activity is apparent during the later stages of the process of osteon formation, when reactive new bone is normally converted to structural bone. The movement of calcium from gut to plasma to bone therefore declines in rate, and significantly greater than normal amounts of calcium move from the plasma to the urine and feces. In young adults, the daily net absorption of calcium, regardless of the total amount (800 mg) in the diet of an adult, is approximately 150 mg (Dent, 1956). Though radioisotope kinetic studies reveal the internal movement of Ca^{++} and a high rate of turnover of bone in the acute stage of osteoporosis, the low daily net absorption of calcium remains to be accurately measured in the chronic stages. Indeed, in the aged osteoporotic, to judge from the total amount of calcium in 24-hour samples of urine, this may be as little as 50 mg.

Twenty-five years ago, the emphasis in research on osteoporosis was on the endocrine control of calcium metabolism. The occurrence of the disorder in patients with acromegaly, hyperthyroidism, diabetes, and hypogonadism was attributed to endocrine imbalance.

It is also possible that endocrinopathy initiates nonspecific degenerative changes and premature aging of bone tissue. Hyperparathyroidism has specific effects on bone and causes osteoporosis by osteoclasis and osteocytolysis, and Cushing's syndrome inhibits the proliferation of osteoblasts and produces bone resorption without osteoclasts, but these conditions may also accelerate the aging process and deterioration of osteocytes. The retention of bone and control of the rate of reduction of bone mass, however, could be determined by an *antiosteoporosis* factor. This seems to be associated with such phenomena as maintenance of blood flow, completion of osteons and development of structural bone, androgen and protein biochemistry, effects of mechanical stimuli of physical exercise upon bone tissue, liver metabolism, and the intracellular physiology of the bone cells.

The point of view set forth above suggests the possibility that treatment of osteoporosis by exercise, with improved diet and injections of sex hormones, may stimulate the rate of capillary circulation of bone and thereby slow the process of aging of bone tissue. Measurements of urinary products of bone breakdown such as hydroxyproline and mucopolysaccharides are now being made to estimate the rate of aging of bone and to evaluate current methods of treatment of osteoporosis.

Summary

1. Two cases have been added to six previously reported cases of osteoporosis, investigated by metabolic balance, radioisotope, and tetracycline technics. The data suggest that the metabolic functions of the skeleton are performed by the 1 per cent of the bone that is reactive or exchangeable, and that this is not significantly abnormal in patients with osteoporosis. The deficiency is in the malfunction of the 99 per cent that is the nonreactive, nonexchangeable, stable, or structural bone.

2. Twenty-five survivors from a group of 100 previously reported aged women (average age 85) were reexamined after 5 years. Three cases had osteoporosis (as previously determined by collapsed vertebrae), further fractures, and significant progress of the disease.

Twenty-two cases, previously free of fractures, did not acquire collapsed vertebrae, and had only a physiologic rate of decline in bone density. These observations suggested that spontaneous collapses of vertebrae are indicative of structural *bone failure,* and this is the cardinal sign of osteoporosis. Bone failure distinguishes the disorder from physiologic bone atrophy of the aging. Bone failure occurs when bone mass declines more rapidly than normal to 50 per cent of the quantity that is found in young adult women.

3. The morphologic and biochemical indices of aging are qualitatively the same, but quantitatively more extreme in osteoporotics than in nonosteoporotics. Many osteons stop growing and calcifying and develop a sclerotic inner margin before they are one-half closed. Bone cells retract, enlarge the lacunae, and disappear in the outer and interstitial lamellae that are farthest from the blood vessel. The matrix becomes more basophilic, metachromatic, and irregular staining. These morphologic and histochemical changes are unspecific, but are approximately 30 to 50 per cent more extreme during middle-aged life in patients with severe osteoporosis. As much as 50 per cent of the bone tissue in the tibia may be dead.

4. The recent literature may be interpreted to propose a unifying hypothesis that extreme dietary deficiencies of calcium and protein, various endocrinopathies, and many degenerative vascular system disorders produce premature or accelerated aging of bone, and that osteoporosis appears as a result. The conditions responsible for retention of bone or the maintenance of an *antiosteoporosis factor* that regulates the physiologic rate of aging of the skeleton are possibly related to the genetic constitution of the individual, but otherwise are entirely unknown.

5. A regimen of vigorous constitutional exercises, including deep breathing, bicycling, swimming, and hiking, should be added to medical or orthopedic treatment to improve the circulation and retention of bone in the aging and osteoporotic skeleton.

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Microradiography of Bone Resorption

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FOR a normal skeleton to be maintained there must be a balance between the addition and the removal of bone tissue. This turnover of bone may be taking place rapidly as in a growing individual, or slowly as in young adults, but whatever the rate, the balance must be maintained to result in the compact, well calcified tissue of a normal skeleton. A number of abnormalities of the skeleton are the result of an upsetting of this balance in turnover of bone; there is an increase or decrease in removal of tissue without a corresponding increase or decrease in addition of bone, and the result is a change in the gross structure of the bone, generally a loss of tissue, which seriously affects its supporting function. If the abnormality existing in various bone diseases is to be understood, the immediate cause of the upset in the balance must first be found, and this may be done by measuring the amount of bone formation and bone resorption and also measuring the extent of decrease in mass of the tissue, or its porosity, a figure which will indicate for how long the balance has been upset.

The Measurement of Bone Turnover

A number of attempts have been made to measure bone formation by equating the amount of new bone laid down in the skeleton at

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any time with the amount of mineral being added at that time (Bauer *et al.*, 1955; Heaney and Whedon, 1958). It is most important to distinguish between these two processes. Mineral metabolism involves the movement of calcium and phosphorus between the body fluids and the bone and can be going on actively in a skeleton that is completely inert in terms of the formation and resorption of tissue. Bone turnover refers to actual addition and removal of tissue to and from the skeleton. Radioactive isotopes of calcium, radium, and strontium, when injected or ingested *in vivo* in man, will be taken up in new bone as it is mineralized, and for this reason their retention by the skeleton has been measured by external counting techniques as an indication of bone formation. However, these elements are also firmly retained by fully mineralized nongrowing bone by a mechanism not associated with the laying down of new bone or even necessarily with net addition of mineral (Arnold *et al.*, 1960; Marshall *et al.*, 1959). Quantitative measurements of this fraction in radium-burdened bone have indicated that it is a significant part of the body burden; in 16 individuals measured, an average of 47 per cent of the total amount was in this diffuse fraction and not associated with new bone formation (Rowland, 1960).

Tetracyclines, which are incorporated into mineralizing bone (Milch *et al.*, 1957), have also been used in an attempt to measure bone formation in small bone samples (Urist *et al.*, 1962), but since they have a distribution in bone similar to that of calcium isotopes (Harris *et al.*, 1962), these results must suffer from the same difficulty of interpretation as the data from calcium and strontium retention studies. A preliminary experiment (Jowsey, 1962) involving microdissection of human biopsy samples labeled with tetracycline has shown that 24 hours after injection or ingestion, only 31 to 62 per cent of the total amount of the dose is associated with formation of bone, 38 to 62 per cent being deposited in nongrowing areas. Investigations into the retention of radioactive isotopes of sulfur and carbon that label the organic fraction of bone would suffer from the same difficulty of interpretation, as they too have a diffuse component not associated with bone formation (Kent *et al.*, 1956; Bloom *et al.*, 1947).

Since it has so far been impossible to separate these two fractions

in *in vivo* procedures or in chemical analyses of whole biopsy samples, there must always be uncertainty in these methods as to the proportion of the retained isotope measured that reflects bone formation.

The most successful attempts to measure bone formation have consisted of sampling small areas of growth in sections of bone from specific sites in the skeleton (Jowsey, 1960; Frost and Villanueva, 1961). Microradiographs of undecalcified sections are perhaps more informative than the more conventional decalcified sections, since they demonstrate the distribution of mineral in the tissue besides showing a most characteristic appearance in areas of formation and resorption. It is possible with tetracyclines (Vanderhoft *et al.*, 1962; Harris *et al.*, 1962) or a bone-seeking radioactive isotope (Jowsey *et al.*, 1953; Plonlot, 1960) to see with the aid of a microscope where growth has taken place, as the new tissue will be marked by a band of the fluorescent material or the radioisotope, or will lie between two bands if the label is given twice at different times. By comparing such areas of growth with a microradiograph of the same section, the characteristic appearance of new bone formation has been established as an area of low density with new lamellae lying parallel to a smooth surface (Fig. 1); most characteristic is the continuous decrease in density right up to the edge of the bone. A bone surface that has recently been growing but has stopped will show a sclerotic ring adjacent to the haversian canal (Fig. 3). Measurements of bone formation may be made using microradiographs of unlabeled material.

It becomes apparent on looking at such measurements in bone from normal elderly people and in specimens of some bone disorders that, generally, there is no great variation in bone formation. It would seem that it is increases or decreases in resorption that are important in producing the gross changes seen in abnormal bone, so that attempts to evaluate bone formation only would not measure the cause of the upset in the balance of bone turnover. Resorption must be measured.

Bone resorption is unfortunately more difficult to characterize than bone formation, since it consists of the removal of tissue, and it is possible only to deduce from circumstantial evidence what a

FIG. 1. Microradiograph of a cross section through the mid-shaft of the femur of a 2-year-old male. Bone formation is occurring at *a* and *b* while resorption is occurring at *c* and *d*. There are a number of areas of interstitial bone containing enlarged lacunae (*e* and *f*) which are characteristic of woven or rapidly growing bone. (× 20.)

surface from which bone is being removed looks like in, for instance, a microradiograph of a bone section. This evidence comes from a number of sources. The periosteal surface of the metaphysis of a long bone from a growing animal is an area where removal of tissue must be occurring as the diameter of the shaft becomes smaller. In addition, histological preparations show osteoclasts on such surfaces, and osteoclasts have been observed to resorb bone in tissue culture (Goldhaber, 1960). Such a periosteal surface must therefore be undergoing active resorption. This surface appears in a microradiograph as an irregular, highly calcified surface.

The resorption of bone has also been associated with the deposition of radioactive isotopes of the rare earths and, in particular, of yttrium. Y^{91} has been shown to be localized *in vivo* in areas where

bone is being removed in the process of remodeling in the metaphysis, and in large irregular cavities in cortical bone (Jowsey *et al.*, 1956). Further studies (Jowsey *et al.*, 1958; Neuman *et al.*, 1960) have shown that the deposition of yttrium on the surface of hydroxyapatite is associated with citric acid production or a change in pH which in turn is produced by injections of parathyroid hormone, which causes resorption of bone in conjunction with the production of osteoclasts (Martin *et al.*, 1958). Y^{91} may well be a better indication of bone resorption than the presence of osteoclasts, since it depends on the mechanism of tissue resorption for its deposition in bone, the isotope being taken up on bone mineral in areas where the yttrium in the body fluid is rendered diffusible by a local increase in acidity. Though deposition of yttrium probably always indicates resorption and its absence in high concentrations indicates lack of resorption, the absence of an osteoclast does not always mean that no resorption is occurring in that area (Hancox, 1956; Fell and Mellanby, 1952; Urist and Deutsch, 1960). Therefore the deposition of yttrium in bone can be used to confirm the characteristic microradiographic appearance of a resorbing surface which has been established by comparing the microradiograph with the histological preparation. There should be a high correlation between the sites of deposition of yttrium and the microradiographic appearance of resorption; experimental data confirm this. Autoradiographs of bone sections from a young monkey injected with Y^{91} 24 hours before death were compared with microradiographs of the same sections. The sites of concentrated Y^{91} deposition were correlated with the sites in the microradiograph where the highly calcified surface indicated active resorption to be occurring. Accurate measurements on enlarged photographs of the lengths of surface involved showed that the yttrium deposition and microradiographic appearance of resorption corresponded in 89 per cent of the areas, the remaining 11 per cent being sites either of yttrium deposition which were judged as not showing resorption in the microradiograph or of microradiographic indications of resorption with no concentrated uptake of yttrium. The correlation coefficient between the two sets of results was 0.99. If, on the other hand, the results from the auto-

radiographic measurements of resorption and the microradiographic measurements of resorption are compared directly, paying no attention to whether the measurements correspond, there is a 99.99 per cent similarity between the results. Therefore, assuming the results from the yttrium autoradiographs to be correct, the results from the microradiographs will be 99.99 per cent similar numerically though they will include 10 per cent mistakes. This correlation between the deposition of yttrium and the microradiographic appearance of high density confirms the criteria established for bone resorption by comparison with the presence of osteoclasts in a histological preparation, and also the quantitative data suggest that very nearly 100 per cent of all resorption surfaces are being measured by this method.

These correlative data show that a bone surface that is undergoing resorption appears in a microradiograph as an irregular, crenated, highly calcified surface with the lamellae lying at all angles to the surface rather than around the cavity (Fig. 1).

It is most important to distinguish between a bone surface that is actively being resorbed and appears microscopically crenated and irregular and one where resorption has been taking place but has ceased. The latter surfaces are highly calcified and irregular and have the lamellae running at angles to the surface, like areas of active resorption, but are microscopically smooth and have a narrow rim of high mineralization immediately adjacent to the vascular cavity.

Apart from the areas of active resorption, yttrium is also taken up diffusely throughout the bone tissue, probably in association with low levels of citric acid, and it is also retained to some extent on the highly mineralized surfaces of completed osteons (Jowsey *et al.*, 1956, 1958). Therefore yttrium and the rare earths probably cannot be used to measure bone resorption with any certainty by methods using *in vivo* external counting or chemical measurements on large pieces of bone, because, as with the alkaline earths, it is not possible to differentiate between the fraction of isotope retained as a result of the localized intense activity of the bone, that is, the resorption of tissue, and the slow metabolic activity that occurs throughout the bone.

Summary

Any investigation of bone may profitably include a measurement of turnover, that is, the addition and removal of tissue from the skeleton. Quantitative measurements of growth and resorption in normal tissue may be compared with those in bone from metabolic diseases and other diseases of the skeleton to discover the immediate cause of the abnormality. A number of methods have been devised for measuring bone formation, the most direct and accurate one being the quantitative estimation of growth in a specific area of a bone sample by the characteristic appearance of the surface of the bone, preferably in a microradiograph of an undecalcified section. *In vivo* administration of radioactive isotopes of calcium and other alkaline earths and of sulfur in animals have made it possible to establish the characteristic appearance of a growing surface as one where the lamellae are of low density and lie parallel to a smooth surface. Bone resorption, which is probably the most important cause of imbalance in tissue turnover, can be identified by comparison with histological preparations showing osteoclasts and with the localization of yttrium and the rare earths, which depend on the mechanism of resorption for their deposition in high concentrations. Resorbing surfaces can be identified in a microradiograph by the presence of highly calcified bone with lamellae lying at angles to an uneven, crenated surface.

The sampling method of studying bone turnover has some disadvantages, the most obvious being that it is not easy to be sure exactly how relevant the results from any one sample are to the turnover in the rest of the skeleton. However, the results from one sample area in different individuals may perhaps be treated as comparative data without reference to the rest of the skeleton. Also samples from different parts of the skeleton will produce data that will indicate the magnitude of the variations to be expected throughout the skeleton. The disadvantages are surpassed by the advantages of being able to measure bone formation and, most important, bone resorption without doubt, and also of being able to investigate local lesions in the skeleton.

Material and Techniques

The method adopted for the present study of bone turnover was the quantitative evaluation of bone formation and resorption in microradiographs of bone sections. Both normal and pathological material was used in this study. Normal material was collected from accident cases and sudden deaths from heart attacks. Gross x-ray pictures were made of 1.0-cm slices through the 5th lumbar vertebra to determine whether any osteoporosis was present in the normal individuals; if such x-rays showed abnormally low density, these individuals were excluded from the study. Pathological material came from individuals with bone diseases, in particular osteoporosis. This group consisted of 10 individuals with x-ray evidence of osteoporosis such as decreased density of the skeleton and crush fractures of the spine; about half of the group had also fractured the neck of one femur. The specimens were taken from the mid-shaft of the femur, the neck of the femur, the iliac crest, and the 5th lumbar vertebra, though the results reported here are mainly those from the mid-femoral shaft.

The specimens are fixed in 70 per cent alcohol, dehydrated in absolute alcohol for 2 or 3 days, and then put in unpolymerized methyl methacrylate monomer for 24 hours. The specimens are then placed in semipolymerized monomer and the polymerization is completed by heating at 32°C for 2 to 3 days. The blocks are trimmed on a bandsaw and sections 100 microns thick are cut on a commercial milling machine using a circular steel saw. The sections are further ground, if necessary, until they are 100 ± 2 microns thick, and contact microradiographs are made using Eastman 649-O spectroscopic plates and an x-ray beam from a copper target. The microradiographs are inspected with a microscope and the areas of bone formation, resorption, and inactivity identified. Quantitative measurements are made by making a print at a magnification of 30 times, and since formation and resorption of bone are activities that take place exclusively on the surface, the lengths of all bone surfaces that are undergoing formation or resorption or are inactive are measured with a map measure. The results are expressed as the length of surface involved in either formation or resorption in a certain area

of a section, as a percentage of the inactive surface in that same area. A standard sample area in each site is analyzed. In the mid-shaft of the femur one-third of an entire cross section constitutes the sample area, one-quarter of the area being taken from each of four quadrants. Analysis of smaller samples produces data with considerably less significance.

Measurements of the porosity of the bone are also made. In cortical bone from the femur this porosity measurement consists of the percentage of osteons which are less than two-thirds closed; that is, the diameter of the central canal is more than one-third the diameter of the whole osteon up to its cement line. The thickness of the cortex in the specimens is also measured.

Summary

Undecalcified bone sections are cut from normal and pathological material and microradiographs made. Bone formation and resorption are identified and quantitative measurements made on photographic enlargements. Bone porosity is also measured.

Discussion of Results

Resorption

Resorbing surfaces are obviously places of great metabolic and chemical activity and are in intimate contact with the body fluids as indicated by electron microscope work (Scott and Pease, 1956; Cameron and Robinson, 1958) and the uptake of the rare earths (Jowsey *et al.*, 1958). Calcium[45] and tetracycline are also deposited on these surfaces for a short time (Fig. 2), possibly by an exchange mechanism, and may account to some extent for some of the skeletal retention found at short intervals after an injection of radioisotopes such as Ca^{47} in areas of fracture or in osteoporotic bone, where a great deal of resorption is known to be occurring. Areas of particularly active resorption appear microradiographically as very irregular surfaces with numerous uneven projections into the wall of the cavity. This bizarre resorption was first described by Rowland *et al.* (1959) in microradiographs of radium-burdened bone, but has since been found in almost every bone disorder involving rapid re-

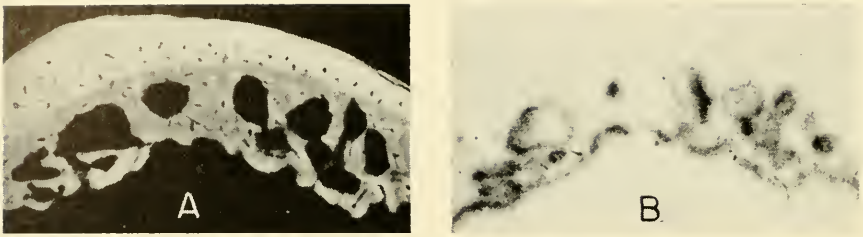

FIG. 2. Microradiograph (A) and Ca^{45} autoradiograph (B) of a cross section through the mid-shaft of the femur of an adult rabbit with cortisone-induced osteoporosis. The animal was killed $1\frac{1}{2}$ hours after Ca^{45} injection, and the isotope is heavily concentrated in areas of bone resorption. (× 22.)

sorption, such as osteoporosis and renal failure, and also occasionally in normal bone (Fig. 3). Despite these indications that there may be different rates of resorption in different areas, in the quantitative measurements it has been supposed that an average value is valid.

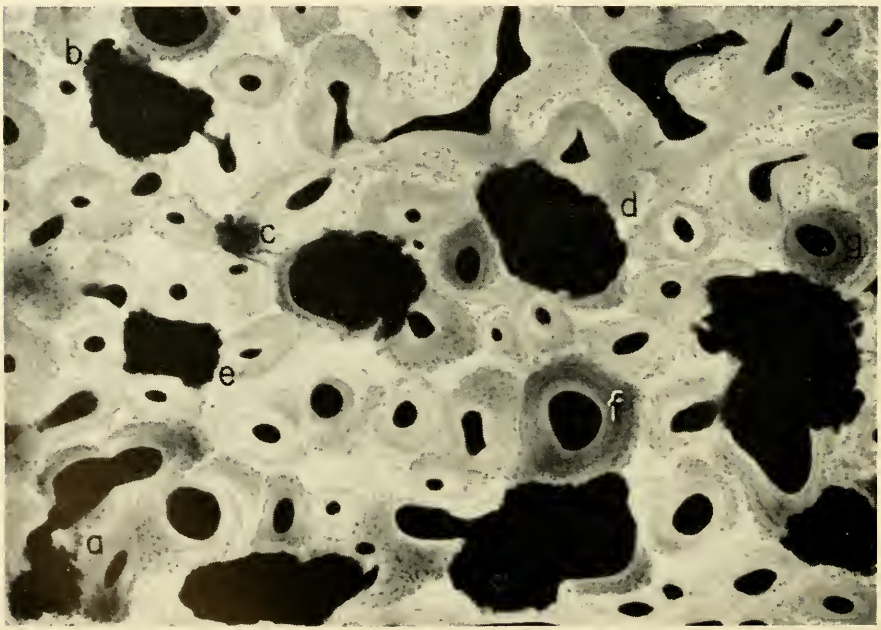

FIG. 3. Microradiograph of a cross section through the mid-shaft of the femur of a 15-year-old female with renal failure, showing the irregularity of surface characteristic of rapid resorption at *a*, *b*, and *c*, and more normal resorption at *d* and *e*. At *g* and *f* are two recently formed osteons with sclerotic rings characteristic of cessation of new bone formation. (× 22.)

Quantitative Measurements

Normal. In order that abnormality may be appreciated, the normal state of affairs must first be described. It is always a little difficult to choose criteria for what shall be considered normal and what abnormal. Since one of the main purposes of this study was to compare normal with osteoporotic bone, certain individuals were rejected from the normal group because a gross slab x-ray through a 1-cm-thick longitudinal slice of the 5th lumbar vertebra showed definite porosis; only those showing unremarkable density in this x-ray were retained as normals. This normal group shows the characteristic pattern of bone turnover that has already been demonstrated (Jowsey, 1960), with the exception that, as a result of excluding those individuals that showed loss of bone mass in the spine, there was less increase in the amount of resorption in older individuals. From Fig. 4 it seems that from the age of 40 onward the level of resorption is above that of formation, but that the difference does not continue to increase after the age of 70. The result of this imbalance is visible in the values for porosity; that is, the number of osteons less than two-thirds closed is increased, while the thickness of the cortex decreases. These graphs do not paint the whole picture: from the microradiographs themselves (Fig. 5) it can be seen that there are generally a greater number of incompletely mineralized osteons in the older individuals and that the increase in number of osteons less than two-thirds closed appears to be the result of a preponderance of resorption cavities which have become only partly filled in with new bone.

Resorption and formation rates. The actual addition or subtraction of tissue mass to or from the bone can be calculated by multiplying the values for surface activities by the rate of formation or resorption which is seen in the thickness of new tissue in a cross section. The results reported here assume that the rates of bone formation and resorption are similar and that they do not vary a great deal without leaving an indication in the microradiograph. Measurements have been made of the rate of new bone formation in cross sections of cortical bone using tetracycline markers (Frost,

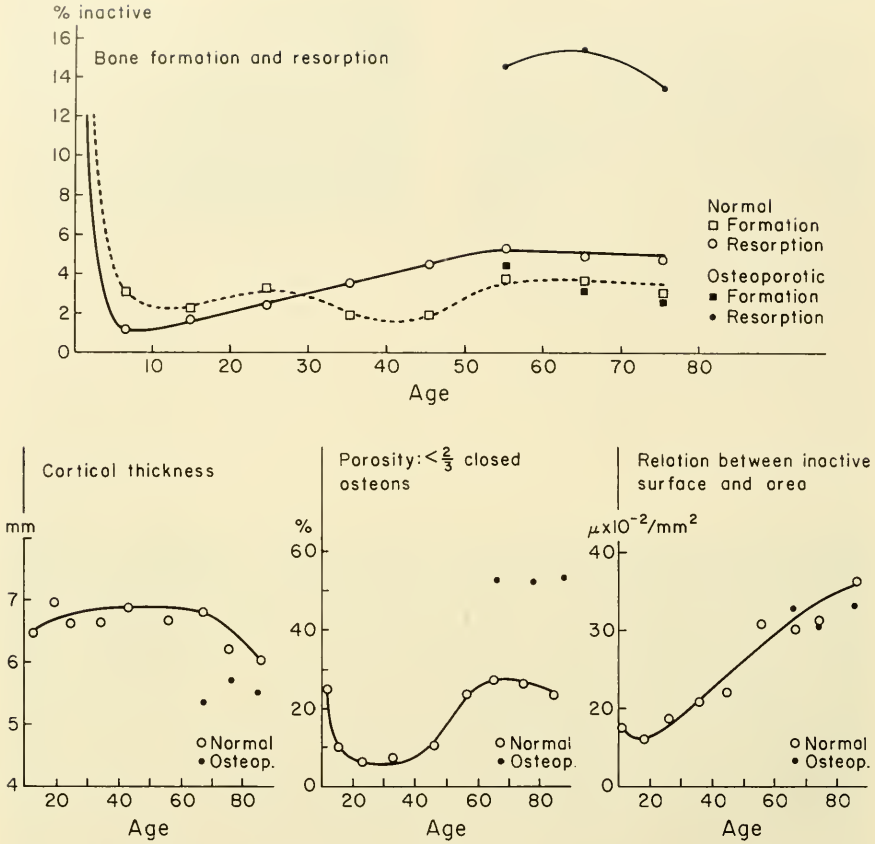

FIG. 4. Turnover data from the mid-shaft of the femur of normal and osteoporotic individuals. See text for details.

1960), and the average value is 1 micron per day. Resorption rates cannot be directly measured, but since a mass of bone that is not losing or gaining tissue, such as cortical bone in a normal 20- to 30-year-old human, shows approximately equal lengths of surface involved in resorption and in formation, it is probably not incorrect to assume that the rate of resorption is approximately equal and also averages 1 micron per day. However, rates of resorption and formation must vary from place to place and time to time. Formation is somewhat more rapid when an osteon is just beginning to be laid down, and the rate becomes slower as the osteon is com-

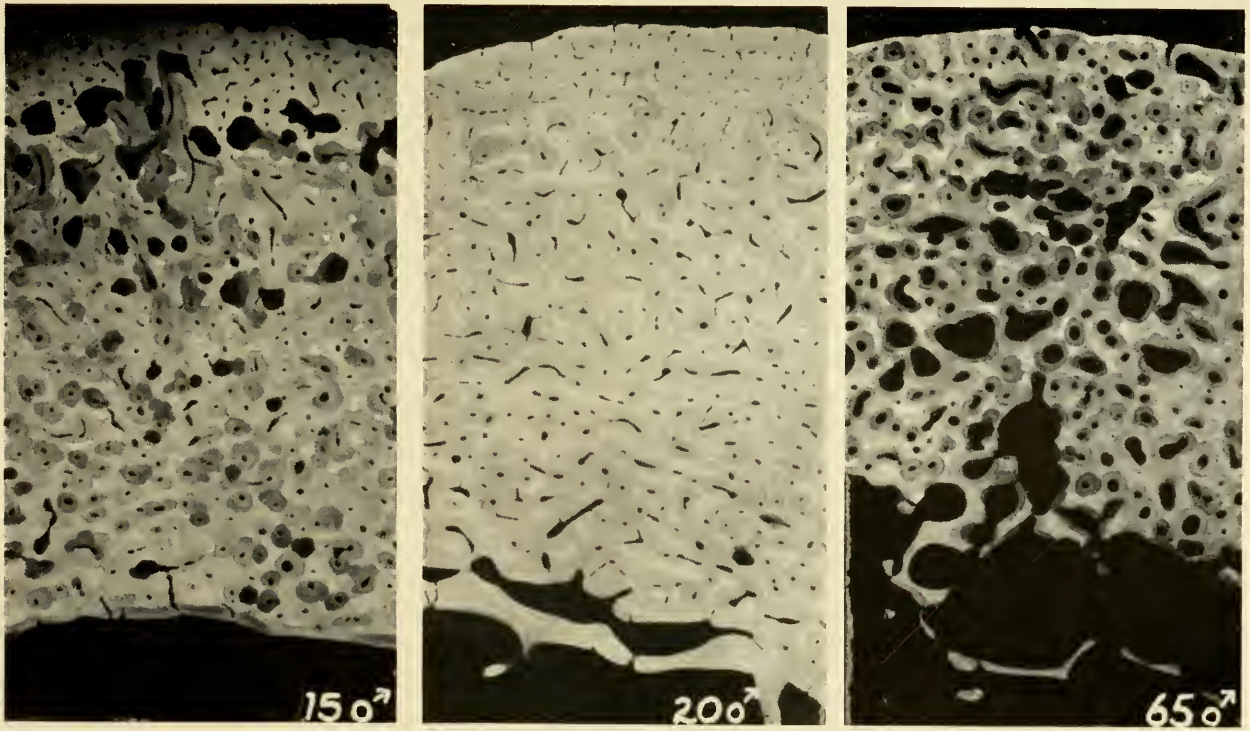

FIG. 5. Microradiographs of cross sections through the mid-shaft of the femur of normal individuals of different ages, to show the change of structure and variations in mineral density. (× 10.)

pleted. As large resorption cavities are more frequent than small ones, and as the average length of surface per cavity is higher for resorption than for formation, it is probable that resorption also takes place more rapidly in a new, small resorption cavity than in an older, large one. However, despite the local variations in the rates of formation and resorption, an average figure should be valid, and measurements of the length of surface occupied by resorption and formation should also be valid and refer to actual bone turnover. The measurements can be converted to mass of tissue added to or removed from bone if one assumes the figure of 1 micron per day for the rate of formation and resorption to be correct and knows the relation between the amount of inactive surface and the area of the sample (Fig. 4) ($20\ \mu \times 10^{-2}/mm^2$ in 20- to 40-year-old individuals and $30\ \mu \times 10^{-2}/mm^2$ in 60- to 80-year-old individuals). This increase in the amount of surface per unit area of bone means that if resorption and formation are to be considered in terms of area or mass rather than surface, the actual values will show a greater increase in old as compared with young adults. In other words, from the graph (Fig. 4), resorption in young adults is $2\ \mu/100\ \mu$ of inactive surface, or 1.5 $mm^3/100\ mm^3$/year, which is balanced by similar values for bone formation. In older individuals the value in terms of length of surface is 4.8 per cent and in terms of mass it is 5.2 $mm^3/100\ mm^3$/year, while bone formation is 3.3 per cent in terms of length of surface and 3.6 per cent per year in terms of mass. Therefore, at this rate a piece of cortical bone should lose 16 per cent of its mass over a 10-year period, a figure that is consistent, as far as can be judged, with the values for porosity and cortical thickness.

Osteoporosis

In comparison with the normal series, the amount of bone formation in osteoporotic bone seems not to be different from the levels of formation found in normals of similar age (Fig. 4). However, 2 out of the 10 cases showed remarkably low values, and these had been bedridden for 2 to 12 months before autopsy; there seems to be an effect of partial immobilization on bone formation. The levels

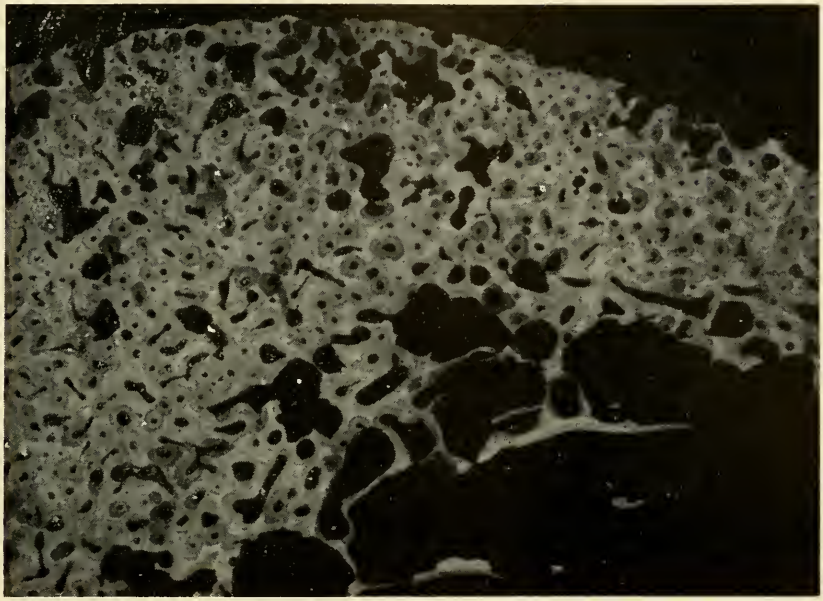

FIG. 6. Microradiograph of a cross section through the mid-shaft of the femur of a 67-year-old female with osteoporosis. There are a large number of resorption cavities and a high proportion of incompletely closed osteons. (× 11.)

of bone resorption are very much higher in this group than in the normal series (Fig. 6). In older individuals there appears to be some equilibrium, and the amount of resorption decreases toward normal values while the levels of bone formation remain the same. The porosity figures are significantly higher in the osteoporotic group than in the normal series, which is to be expected since the porosity represents the cumulative effect of the difference between the levels of resorption and of formation. The values for cortical thickness are generally lower than normal. It would seem, therefore, that osteoporosis is the result of increased resorption of bone, which in the mid-shaft of the femur appears to be a factor of 2 or 3 times above normal values. These differences are exaggerated in the iliac crest, both in the osteoporotics and in the normal series, and for this reason the iliac crest is probably a better biopsy site.

Paget's Disease

Grossly this abnormality varies in its appearance from one of decreased density to one of sclerosis, and the microradiographic appearance of three stages—porosis, sclerosis, and presarcomatous—has been described by Kelly *et al.* (1961). Figure 7A demonstrates the porotic stage, where bone resorption is going on most actively at levels 15 times above the normal level. A higher magnification (Fig. 7B) shows the characteristic mosaic appearance and great variation in degree of mineralization and also the very irregular surfaces of the bizarre resorption cavities associated with rapid disappearance of tissue. At short intervals after injection of tetracycline such irregular surfaces are strongly labeled, indicating particularly high metabolic activity. Quantitative values for formation and resorption are in the order of 20 times the normal levels.

Hyperparathyroidism

Early stages of hyperparathyroidism appear microradiographically like osteoporosis. However, advanced hyperparathyroidism accompanied by a much elevated blood calcium level results in calcification of the fibrous connective tissue in the haversian canals and resorption cavities (Fig. 8). Both bone resorption, which is a factor of 10 or more times above the normal level, and bone formation, which is also increased, appear to be going on rapidly.

In both Paget's disease and hyperparathyroidism the increased resorption levels may result in generalized or local porosis and eventually fracture of the bone.

Osteogenesis Imperfecta

Measurements of bone formation and resorption show that in osteogenesis imperfecta there is both a reduction in formation to less than a third of its normal value and a threefold increase in the amount of resorption. It would seem that this situation has existed for some time, since there is a considerable amount of unremodeled primary periosteal and endochondral bone with large resorption cavities, and the cortex is very thin (Fig. 9). The bone itself appears

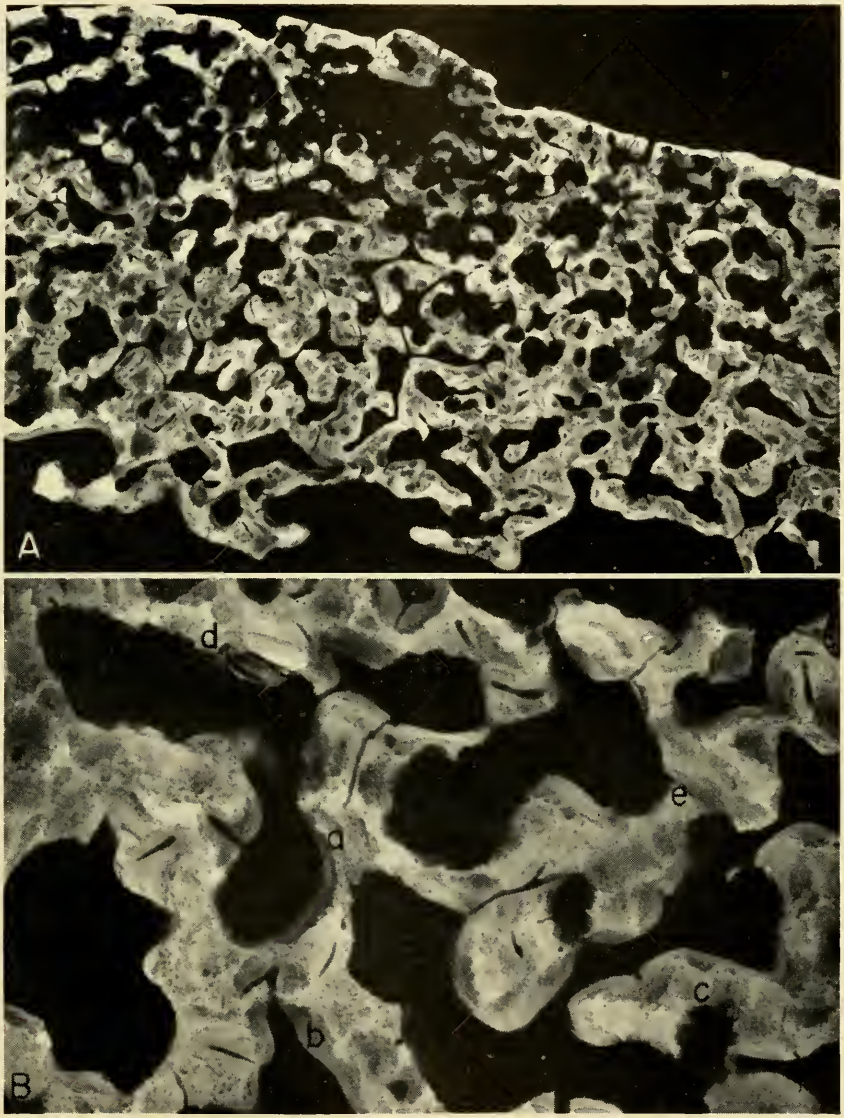

FIG. 7. A, low-power (× 9), and B, high-power (× 30) microradiographs of a cross section of the femur of a 78-year-old female showing the porotic phase of Paget's disease with the large number of spaces, mosaic pattern of mineralization and bone formation (*a* and *b*), and resorption (*c*, *d*, and *e*) taking place on nearly all surfaces (formation 50 per cent, resorption 140 per cent).

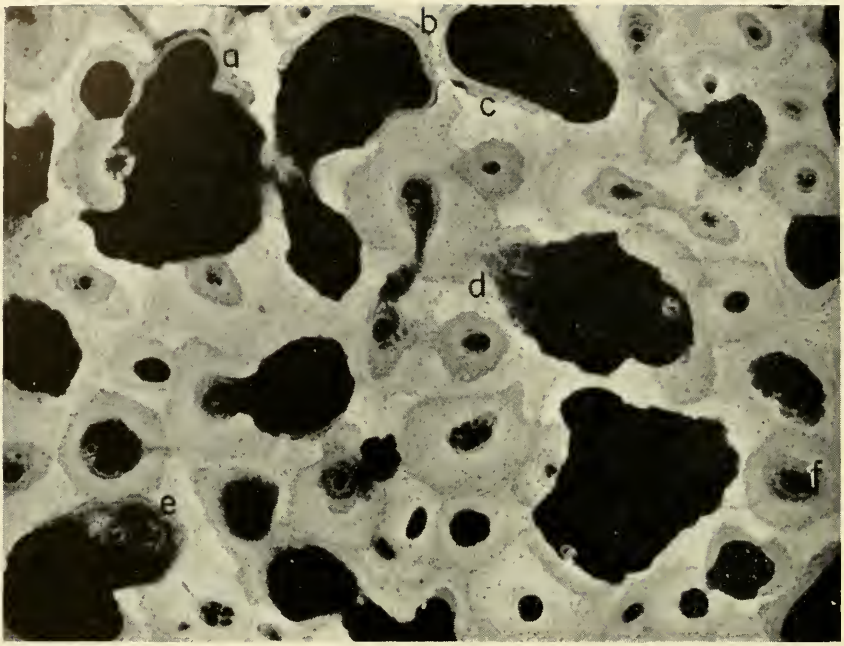

FIG. 8. Microradiograph of a cross section through the femur of an individual with hyperparathyroidism, showing recent new bone formation at *a*, *b*, and *c*, and mineralization of connective tissue in haversian spaces at *d*, *e*, and *f*. Most bone surfaces are in the process of resorption. (× 22.)

normal. In osteogenesis imperfecta, abnormal levels of both formation and resorption contribute toward the decreased mass of tissue.

Rickets

The microradiographic appearance of rickets is most unusual. The gross appearance (Fig. 10A) is one of porosity with many areas of decreased density; the high-power appearance (Fig. 10B) shows the decreased mineral density of the cement lines surrounding the osteons and the lack of mineral around the osteocytes and their canaliculi. It is the enlarged canaliculi that are mainly responsible for the unusual appearance, and whether this represents incomplete mineralization as the bone is laid down or demineralization and

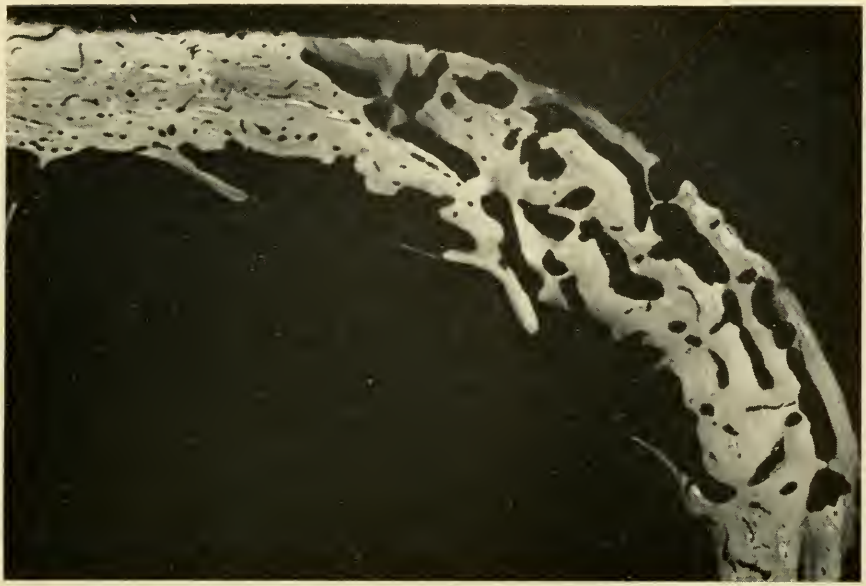

FIG. 9. Microradiograph of a cross section through the femur shaft of a 12-year-old male with osteogenesis imperfecta. The cortex is thin and there is a high proportion of unremodeled primary bone. Compare with 15-year-old male in Fig. 5. (Formation 9.0 per cent, resorption 54 per cent.) (× 6.)

leaching out of mineral is unsure. The surfaces of most osteons are lined with uncalcified osteoid tissue of great thickness. The presence of osteoid tissue does not always indicate bone formation; in rickets and hypoparathyroidism the low blood calcium levels may cause failure of mineralization of osteoid tissue that has been laid down some time ago, and this may be true in other abnormalities or even in normal bone. It is worth while to point out the differences between the appearance of the osteocyte lacunae in rickets and that of the somewhat irregular and enlarged lacunae of the osteocytes in rapidly growing or woven bone (Fig. 1), where the walls of the lacunae are smooth and sharply defined and the canaliculi normal in diameter in contrast to the fuzzy appearance of the osteocyte lacunae and involvement of the canaliculi seen in rickets. This appearance is also seen in osteomalacias and in the early development of Paget's disease.

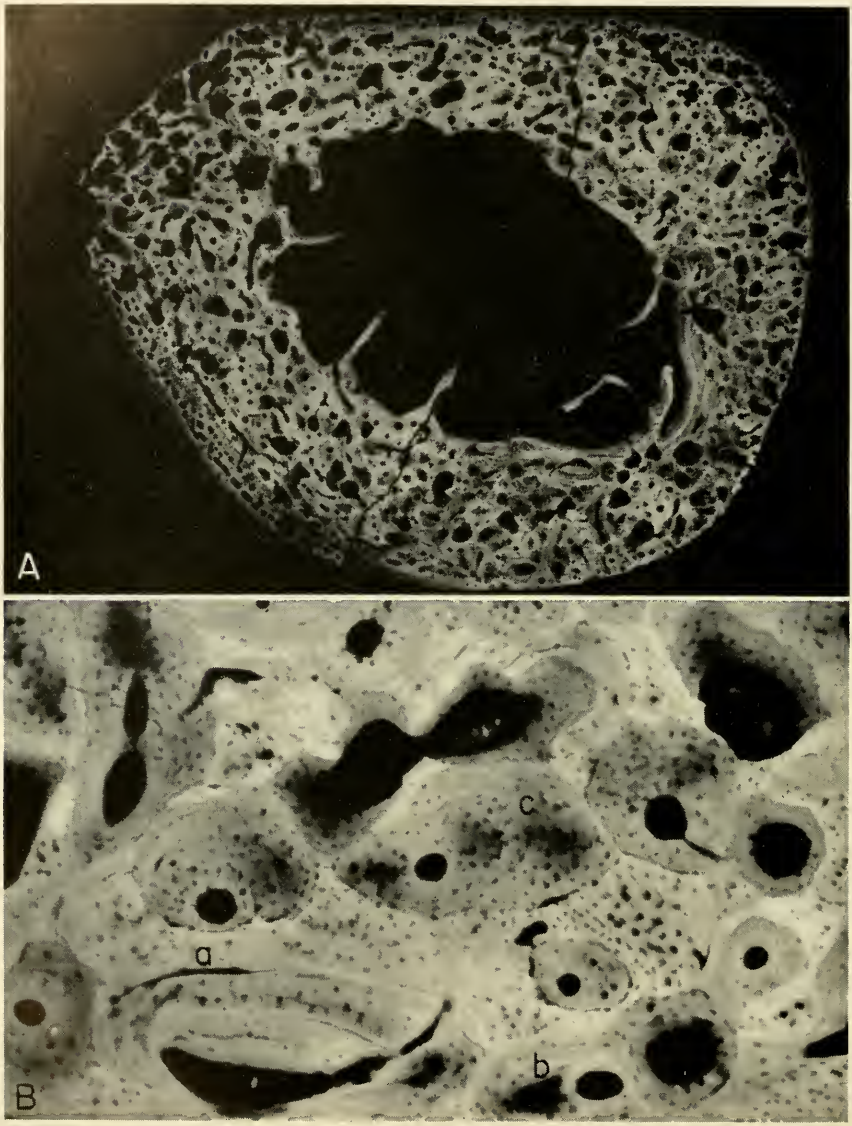

FIG. 10. A, low-power (× 9), and B, high-power (× 30) microradiographs of a cross section through the fibula of a 9-year-old male with vitamin D–resistant rickets, showing the porosity, unmineralized cement lines (*a*), and lack of mineral around lacunae and canaliculi (*b* and *c*).

Conclusion

The results have demonstrated some of the facts that can be learned from microradiographic studies of bone and have shown the importance of resorption in both the normal and the abnormal skeleton. The quantitation provides some sort of base line for comparison and makes possible the constructive evaluation of therapy in bone disorders.

Summary

Bone turnover was measured in a defined area by techniques involving semiquantitative analysis of microradiographs. Formation and resorption of bone as well as its porosity were measured and related to the age of the individual, and comparisons were made between normal and abnormal tissue.

It was apparent that in normal individuals an increase in the amount of bone resorption is what produces the porosity characteristic of aging bone. Osteoporotic bone appeared to be an exaggeration of the normal aging process in that the increased resorption caused the porosity.

Bone from individuals with Paget's disease, hyperparathyroidism, osteogenesis imperfecta, and rickets had a distinctive appearance.

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17

Histophysical Studies on Bone Cells and Bone Resorption

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ONE approach to the study of the mechanisms of hard tissue destruction directs attention to possible variations in the tissues themselves in regions preferentially disposed to such destructive processes. In regard to bone, this relationship has frequently been investigated in systemically accelerated resorptive states induced by parathyroid stimulation, or by injection of parathyroid extract. The results of these studies have led, however, to contradictory conclusions.

Under such circumstances, "stable" rather than "labile" bone is said to be preferentially resorbed. "Stable" bone apparently corresponds to older, more heavily mineralized bone, while "labile" bone refers to newer, less completely calcified bone* (McLean and Urist, 1961).

A number of studies indicate, in fact, that under parathyroid stimulation there is selective resorption of older, fully mineralized bone (Woods and Armstrong, 1956; Clark and Geoffroy, 1958; Elliott and Talmage, 1958; Talmage *et al.*, 1959, 1960, 1961). Other data, however, suggest that it is actually *newly* formed bone, in

* Stable bone has also been referred to as "nonexchangeable," "unavailable," "deeper," "well established," and "structural" bone. Labile bone has also been designated "reactive," "exchangeable," and "metabolic" bone.

areas of normally active osteogenesis, which is preferentially removed (Jaffe *et al.*, 1931*a*, 1931*b*; Heller *et al.*, 1950; Talmage *et al.*, 1953; Lotz *et al.*, 1954; Bronner, 1961). The rapid destruction of metaphyseal trabeculae is often noted, and this is a region of relatively *low* mineralization (Weidman and Rogers, 1950; Engström and Bergendahl, 1958; Arnold, 1960).

Indeed, it has been reported that matrix need not be calcified at all to yield to resorption (Carnes, 1950; Follis, 1952), although some studies indicate that uncalcified matrix is refractory to resorption (Weinmann and Schour, 1945; Reitan, 1951), and other experiments indicate that matrix need not be calcified at the time of resorption, so long as it was calcified at some time in the past (Irving and Dale, 1962).

Finally, there are some reports (Engfeldt and Zetterström, 1954) indicating that both old and new bone are removed, following systemic acceleration of resorption; and published microradiograms indicate that resorption may extend through areas with varying degrees of calcification (e.g. Engfeldt and Zetterström, 1954; Sognnaes, 1959; Jowsey, 1960).

In the light of these inconsistent findings, it appeared worth while to summarize a number of previously unpublished observations which bear on the relation of the state of the intercellular matrix to preferential sites of resorption. Furthermore, since it appears to be generally accepted that bone resorption is an active, cellular process (McLean, 1954; Goldhaber, 1961; Talmage, 1962), attention has also been directed to the cellular element in resorption, and to controlling mechanisms which operate at the cellular level.

Materials and Methods

Young rats of the Long-Evans strain have been used throughout. The bones of normal animals have been analyzed by a number of techniques, and have been compared with those of animals in which bone resorption had been accelerated by the prior intraperitoneal injection of parathyroid extract (PTE).* Generally, a total of 75 to

* Injection parathyroid U.S.P., Lilly; in part donated by Eli Lilly Company, Indianapolis, Indiana.

100 units of the extract was administered in 3 or 4 doses over a period of 7 to 8 hours in suckling rats between 5 days and 2 weeks of age at the time of injection. When weanling rats were used (3 to 4 weeks of age), the dose was increased to a maximum total of 200 units administered over an 8-hour period. The costo-chondral junction of ribs, the tibiae (both regions sectioned longitudinally), and the parietal bones of the cranial vault (sectioned coronally) have been examined. Unless specifically stated otherwise, specimens were fixed in Bouin-Hollande solution for 3 days, decalcified in 18.5 per cent versene at pH 7, double-embedded in celloidin-paraffin, sectioned at 5 microns, and mounted in Permount.

The following analyses were carried out on this material.

Histological Stains

Sections were stained with hematoxylin and eosin, chrome hematoxylin–phloxine, Masson's trichrome, Bodian's silver (Gridley, 1960), Gomori's silver (Romeis, 1948; fixation 10 per cent formalin in 95 per cent ethanol for 24 hours), and toluidine blue (fixation in Rossmann's fluid at 4°C overnight; 0.1 per cent aqueous stain, 20 minutes at 45°C, with and without alcoholic differentiation).

Methyl Green–Pyronin

Bones were fixed in 10 per cent neutral formalin for 16 hours, and stained with methyl green–pyronin Y, according to Brachet (Pearse, 1960). Some sections were incubated in a ribonuclease (Worthington Biochemical Corporation) solution (1 mg/ml distilled water) at 37°C for 3 to 4 hours, or in water alone under the same conditions, prior to staining.

Periodic Acid–Schiff

Bones were fixed in Rossmann's fluid at 4°C overnight, and decalcified in 2 per cent nitric acid in 80 per cent ethanol. Sections were spread on 70 per cent ethanol, dried at 37°C, coated with 1 per cent celloidin, and stained with periodic acid–Schiff (PAS). Some sections were incubated in saliva or in water at 37°C for 1.5 hours, prior to staining. All sections were counterstained with hematoxylin, and mounted in Canada balsam.

Supravital Staining

Calvaria only were used. Immediately upon sacrifice, the calvaria were placed in a solution of 0.01 per cent neutral red in 0.9 per cent saline at 37°C for 50 minutes, then were fixed in 1 per cent mercuric chloride for at least 1 hour. The preparations were examined with the dissecting microscope, intact and unmounted.

Microradiography with Ultrasoft X-Rays

Bones were fixed in neutral formalin and embedded in paraffin. Sections were floated onto Kodak spectroscopic plates (649-O) according to the technique of Greulich (1960). Exposure to the ultrasoft (wavelength > 10 A) polychromatic x-rays varied from 45 to 50 minutes at 1.5 kv and 1 ma (copper target). The plates were developed in Dektol (Eastman Kodak) for 5 minutes at 20°C. Sections were floated off the plates before development, remounted on glass slides, and stained with hematoxylin and eosin, or with PAS-hematoxylin.

Microradiography with Soft X-Rays

Bones were fixed in absolute ethanol and embedded without decalcification in methyl methacrylate. Sections were cut on a rotary saw at 250 microns, and ground by hand on abrasive paper to about 15 microns. The sections were then placed on Kodak spectroscopic plates (649-O) and exposed to soft (wavelength < 10 A) polychromatic x-rays for 10 to 12 minutes at 12 kv, 19 ma (copper target and beryllium filter) at a tube-to-film distance of 15 cm. The exposed plates were developed in Dektol for 5 minutes at room temperature. Sections were examined separately in the phase contrast microscope without removal of the embedding medium.

Autoradiography

Animals were injected intraperitoneally at 6 days of age with 5 μc/gm body weight of glycine-H^3 (New England Nuclear Corporation, sp. act. 194 mc/mmole) in 0.1 ml of isotonic saline, and sacrificed at various intervals after injection. Autoradiograms of sections

were prepared using NTB2 liquid emulsion (Eastman Kodak). Following 1 week's exposure, the preparations were developed in Dektol for 2 minutes at 17°C. Some sections were stained with PAS prior to autoradiography. All sections were stained with hematoxylin following development of the autoradiograms.

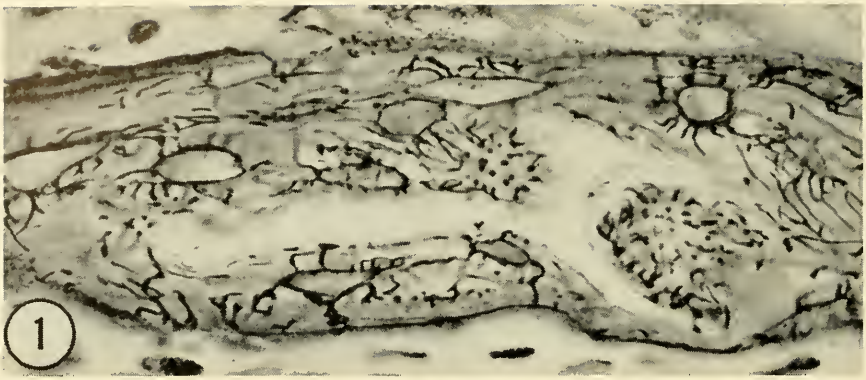
Findings

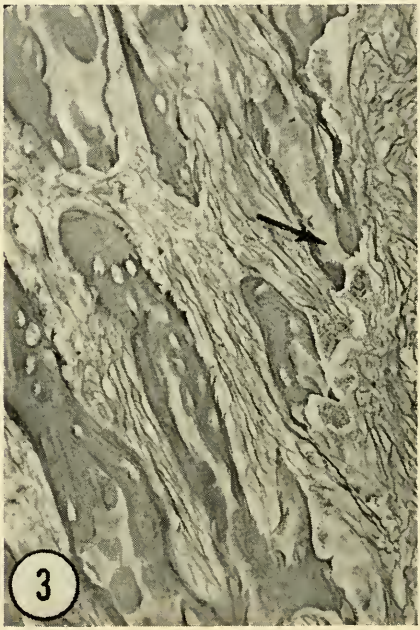
Differences in Degree of Calcification and in Organic Mass

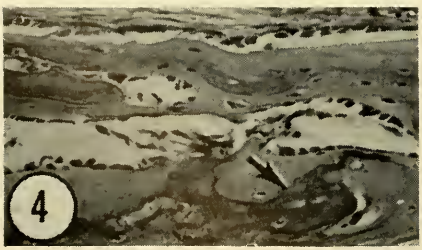
In soft x-ray microradiograms of undecalcified sections, less heavily calcified regions were distinguished from those of greater mineralization by the greater exposure (blackening) of the emulsion in the former, due to the increased permeability of the matrix to the x-irradiation (Figs. 10, 13, and 20). In the immature bones and calcified cartilage of these young rats, such differences are not prominent. Uncalcified matrices of cartilage or of bone (osteoid) do not perceptibly absorb the soft x-rays, and thus were not distinguishable in the microradiograms. They could be detected by comparison of the microradiograms with the corresponding ground sections (Figs. 13 and 14). In ultrasoft microradiograms of dehydrated, decalcified sections, differences in dry (organic) mass content (Greulich, 1961) were reflected in the greater blackening of the underlying emulsion in regions of decreased mass (Figs. 5 and 7).

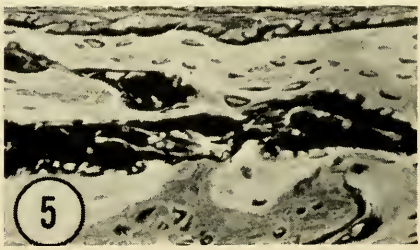
Distinction between Old and New Bone

Differences in the relative ages of different regions of bone may often be deduced from the established sequence of events in normal bone growth. Such differences were objectively demonstrated in autoradiograms of bones from glycine-H^3–injected animals (Figs. 8, 9, and 22). Bone matrix present prior to the time of injection ("old" bone) remained unlabeled, although the walls of osteocyte lacunae within these regions became labeled if they were being remodeled at the time of injection. Bone matrix formed within 4 hours after injection was heavily labeled, and appeared as a prominent reaction band. Matrix formed for several days thereafter was diffusely and progressively weakly labeled (cf. Young, 1962*b*).









FIGURES 1 TO 5

Distinction between the Matrices of Calcified Cartilage and Bone

Calcified cartilage was readily distinguished from bone in ribs and tibiae by the absence of cells (Fig. 1), and by staining differences with all histological procedures used. In ultrasoft x-ray microradiograms, the matrix of calcified cartilage was sharply demarcated from that of bone by its considerably reduced organic mass content (Figs. 6 and 7). However, no appreciable differences were detected in the degree of calcification of these two matrices in the soft x-ray microradiograms prepared from undecalcified sections (Fig. 19).

Distinction between Woven and Lamellar Bone

Woven bone could be distinguished from lamellar bone (most easily in the skull) owing to differences in the matrix which are reflected in increased basophilia, in greater irregularity of the collagen bundles (Figs. 2 and 12), and in affinity for the PAS reaction (Figs. 4 and 22). Furthermore, in woven bone osteocytes were generally larger, closer together, and more irregularly arranged (Figs. 2, 4, 5, 11 to 14). Woven bone, in contrast to lamellar bone, was also char-

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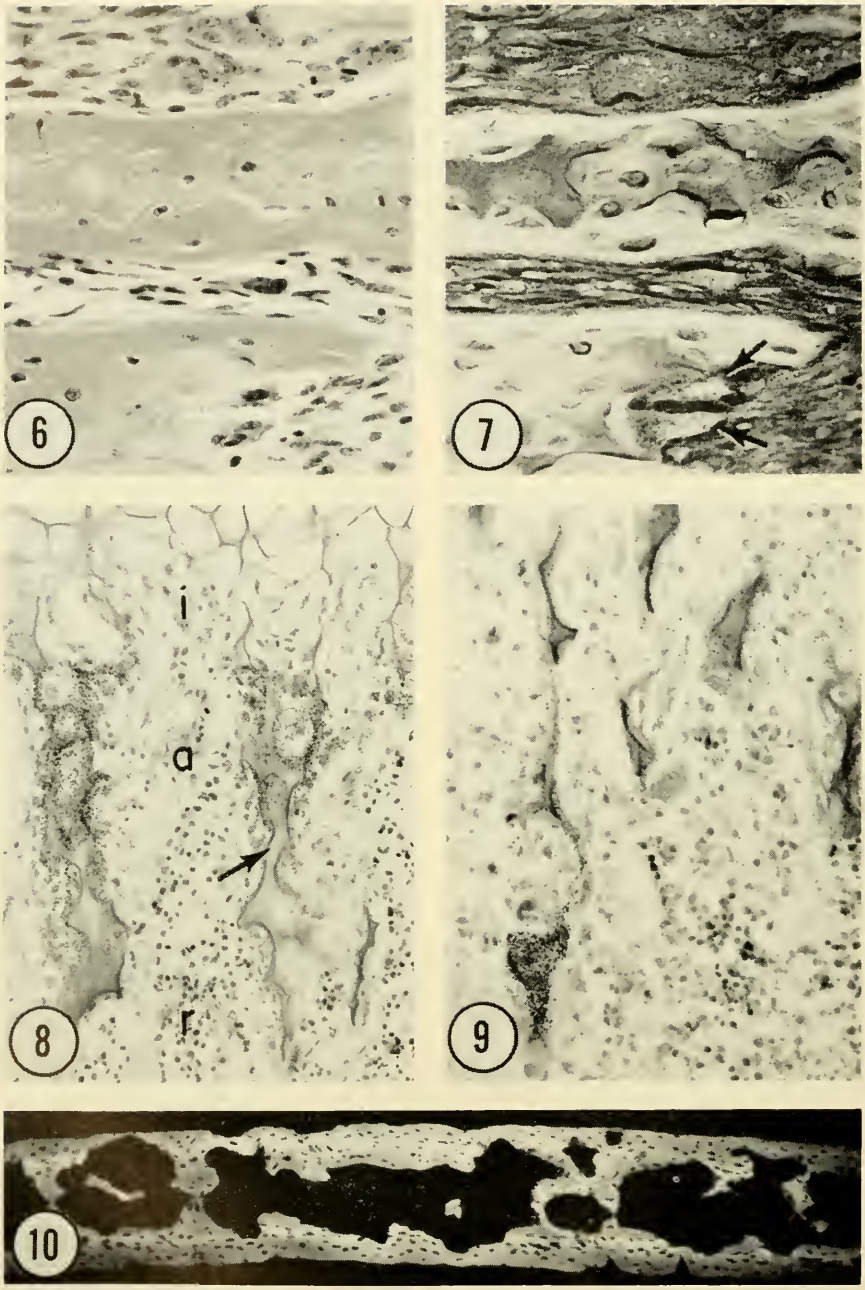
FIG. 1. Metaphyseal trabecula from the tibia of a 10-day-old rat. Note the unstained core of calcified cartilage matrix. The canaliculi of osteocytes situated within the surface coating of bone do not penetrate the cartilage. Bodian's silver. (× 800.)

FIG. 2. Parietal bone of a 26-day-old rat, showing regions of woven (*w*) and lamellar (*l*) bone. Note the differences in the arrangement of collagen fibers and in the distribution of osteocytes in these two types of bone matrix. Gomori's silver. (× 800.)

FIG. 3. Tibial metaphysis of a 26-day-old rat, illustrating normal, subperiosteal resorption accompanying remodeling of the "funnel" region. Note that osteoclasts (right) are engaged in the simultaneous resorption of calcified cartilage (arrow) and bone. Gomori's silver. (× 200.)

FIG. 4. Part of the parietal bone, near the parietal suture, in a 17-day-old rat. A segment of woven bone, stained heavily with periodic acid–Schiff, is indicated by an arrow. The boundaries of the diploic spaces are unrelated to the distribution of either woven or lamellar bone. PAS-hematoxylin. (× 200.)

FIG. 5. Ultrasoft x-ray microradiogram of the region shown in Fig. 4. Note the greater organic mass of lamellar bone as compared with woven bone. (× 200.)



FIGURES 6 TO 10

acterized by a slightly reduced organic mass content (Fig. 5), and by a slightly increased degree of calcification (Figs. 13 and 20).

Sites of Preferential Resorption in Normal Rats

Sites of resorption associated with growth remodeling vary among different bones, and in the same bone at different stages during maturation. However, at the costo-chondral junction of ribs, and in the metaphyseal region of tibiae, resorption not only is particularly rapid, but occurs in a characteristic and similar manner, as judged by the presence of osteoclasts, by the uptake of glycine-H^3 (which is minimal in resorptive areas: Young, 1962*b*, 1963*a*), and by the progressive removal of labeled matrix (formed elsewhere) in these resorptive zones (Figs. 8 and 9). In both ribs and tibiae of these

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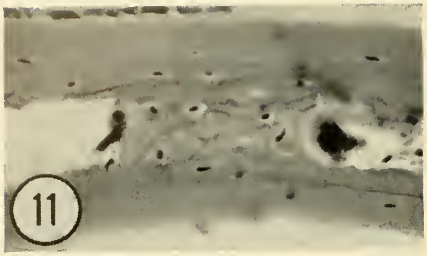
FIG. 6. Tangential section through the shaft of a rib from an 11-day-old rat injected with 100 units of parathyroid extract 1 day prior to sacrifice. Scattered segments of calcified cartilage matrix are present. Hematoxylin and eosin. (× 320.)

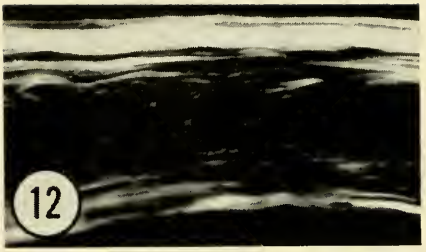
FIG. 7. Ultrasoft x-ray microradiogram of the region shown in Fig. 6. The cartilaginous remnants are readily apparent, owing to their lower organic mass content as compared with that of the surrounding bone matrix. The prominent nucleoli within the osteoclast nuclei (top right) are characterized by high organic mass content. Arrows indicate two osteoclasts (or perhaps two segments of a single osteoclast) engaged in the simultaneous resorption of matrices of differing dry mass. (× 320.)

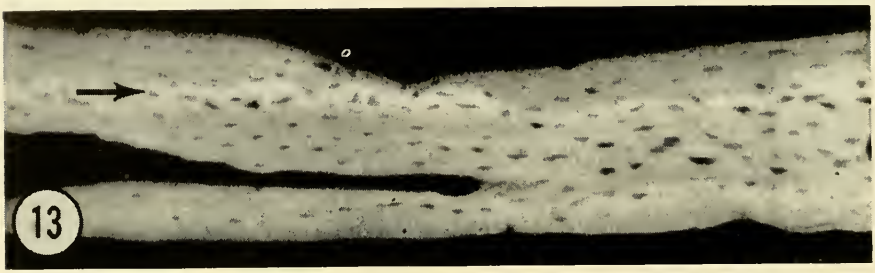
FIG. 8. Tibial metaphysis of a 6-day-old rat sacrificed 2 hours after injection of glycine-H^3. The radioactive material, incorporated in large amounts by osteoblasts engaged in matrix formation, is concentrated in the zone of bone apposition (*a*), and is sparse in the zones of cartilage invasion (*i*) and trabecular resorption (*r*). Calcified cartilage matrix (arrow) remains unlabeled. Autoradiogram, PAS-hematoxylin. (× 200.)

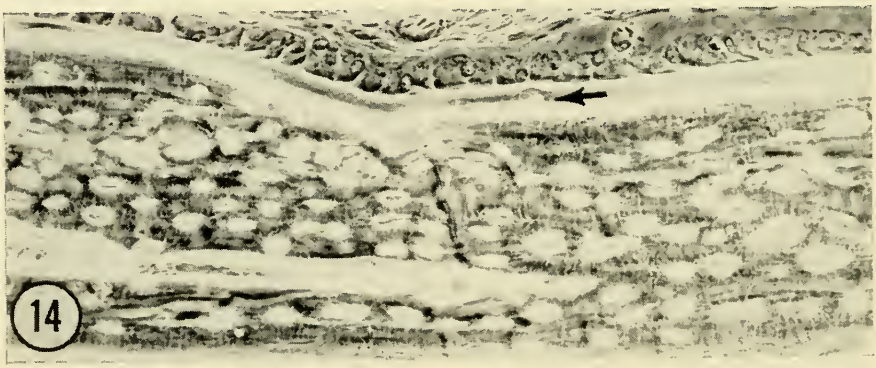
FIG. 9. Zone of trabecular resorption in the tibial metaphysis of a 7-day-old rat sacrificed 32 hours after injection of glycine-H^3. Heavily reactive bone matrix, formed in the zone of bone apposition in the first 4 hours after injection, is now restricted to the distal extremities of the longest trabeculae (lower left). Resorption has removed the major part of the metaphyseal spongiosa which was present 32 hours previously. Autoradiogram, PAS-hematoxylin. (× 320.)

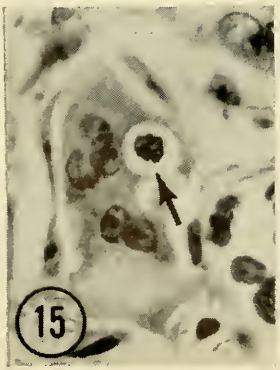
FIG. 10. Soft x-ray microradiogram of a segment of parietal bone from a 23-day-old rat injected with 150 units of PTE 1 day prior to sacrifice. The diploic spaces have been markedly enlarged by resorption, which has proceeded without regard to regional variations in the degree of calcification of the matrix. (× 80.)

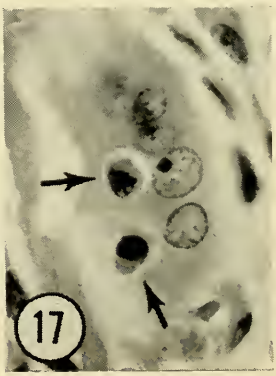












FIGURES 11 TO 17

young rats, there is continued removal of some (but not all) of the calcified cartilaginous intercellular partitions in the zone of cartilage invasion at the distal edge of the growth cartilage. (In the central regions of the metaphyseal trabeculae, in the zone of bone apposition, a thin coating of bone is laid down on those cartilage partitions which escape resorption.) Similarly, in both bones resorption occurs at the distal extremities of the mixed (bone and calcified cartilage) metaphyseal trabeculae in the zone of trabecular resorption, keeping pace with lengthening of the trabeculae, which occurs through vascular invasion of the growth cartilage (Figs. 8 and 9). On the periosteal surface of the metaphyseal regions of these bones, there is a characteristic region of resorption associated with maintaining the "funnel" shape of the tibia, and the curvature of the ribs (Fig. 3).

In the crania of these rats (e.g., the parietal bones) resorption is observed endocranially, just within the peripheral bone margin, and to a limited extent ectocranially, in the central region of the individual bones. These findings, largely derived from glycine-H^3–injected rats (cf. Young, 1962*b*), are consistent with the distribution

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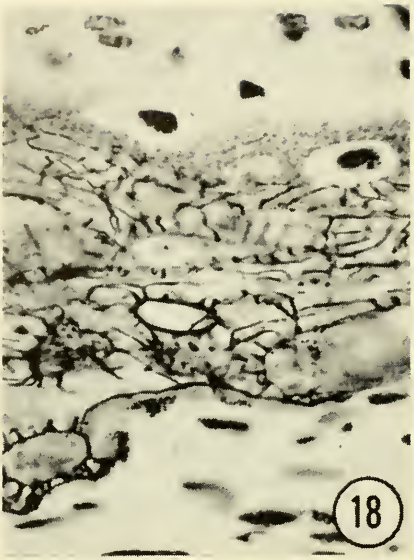
FIG. 11. Parietal bone of a 20-day-old rat, illustrating formation of diploic spaces by resorption in a region where a segment of woven bone lies midway between the inner and outer tables. The osteoclast on the right is engaged in simultaneous resorption of woven and lamellar bone. Hematoxylin and eosin. (× 320.)

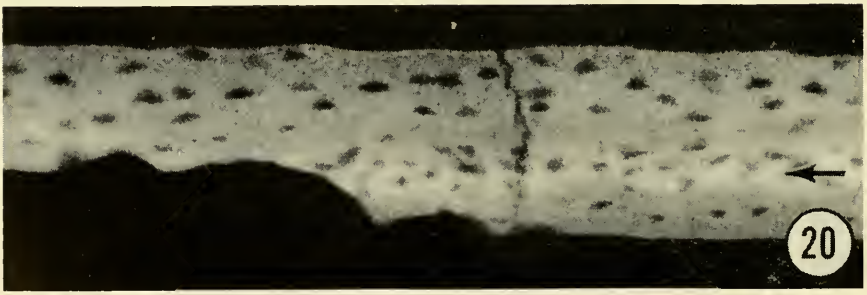
FIG. 12. The same region shown in Fig. 11, photographed in polarized light. The more irregular arrangement of collagen fibers in woven bone, as compared with lamellar bone, is illustrated by the reduced refractility of the former. (× 320.)

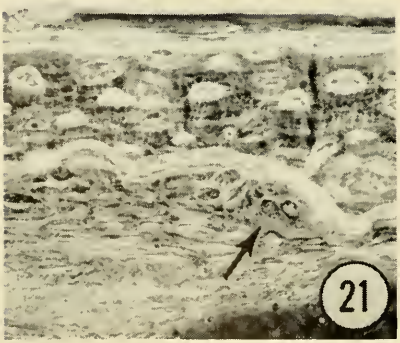
FIG. 13. Soft x-ray microradiogram of a segment of parietal bone from a 23-day-old rat. A thin remnant of woven bone (arrow), characterized by a more irregular disposition of osteocyte lacunae, is slightly more heavily mineralized than the surrounding lamellar bone. Boundaries of the diploic space are unrelated to these minor differences in the degree of calcification. (× 200.)

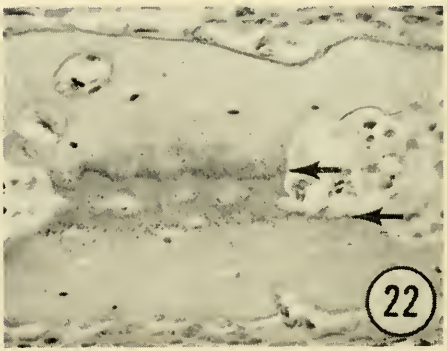
FIG. 14. Phase contrast photomicrograph of the central portion of the section from which the preceding microradiogram was made. A layer of osteoid is present on the upper surface of the bone. The osteoid layer is coated by osteoblasts, and is thick enough to contain an osteocyte (arrow). Note that osteocyte canaliculi are visible within the bone, but not in the osteoid. (× 320.)

FIGS. 15 TO 17. Four cells, apparently phagocytized, are shown within cytoplasmic vacuoles (arrows) in osteoclasts. Chrome hematoxylin–phloxine. (× 800.)









FIGURES 18 TO 22

of osteoclasts observed on the surface of crania stained supravitally with neutral red (cf. Young, 1959). At about 10 days of age and thereafter, resorption becomes prominent within the vascular spaces of the parietal bones in association with the development of the diploe.

FIG. 18. Rib shaft of a 10-day-old rat, stained according to Bodian's silver technique. Osteogenic surfaces (above) are characterized by a granular staining of the matrix. Osteocyte canaliculi are not stained within this new matrix, but are clearly visible in the underlying, older matrix. Resorptive surfaces (below) are sharply outlined, and are further distinguished by the staining of associated canaliculi. (× 800.)

FIG. 19. Soft x-ray microradiogram of the tibial metaphysis of an 11-day-old rat, injected with 100 units of parathyroid extract 1 day prior to sacrifice. Growth cartilage is on the right, marrow cavity on the left. Note the apparent fracture of the shaft (upper left). An increase in resorption in the zone of trabecular resorption (left) has resulted in a shortening of the metaphyseal trabeculae, and a similarly accelerated resorption in the zone of cartilage invasion (*x*) has led to the apparent disengagement of some trabeculae from their normal attachment to the calcified parts of the growth cartilage. (× 80.)

FIG. 20. Soft x-ray microradiogram of a part of the parietal bone of a 15-day-old rat, injected with 100 units of PTE 1 day prior to sacrifice. Endocranial resorption predominates in this region, in which development of the diploe has not yet been initiated. A thin remnant of woven bone (arrow) is slightly more heavily mineralized than the surrounding lamellar bone. Sites of preferential resorption (indicated by the irregular surface, lower left) are unrelated to these differences in calcification. The vertical line (center) results from a crack in the section. (× 200.)

FIG. 21. Phase contrast photomicrograph of the left part of the section from which the preceding microradiogram was made. Note the absence of osteoid, and the presence of an osteoclast (arrow) apparently engaged in resorbing bone of varying degrees of mineralization. (× 320.)

FIG. 22. Parietal bone from an 18-day-old rat, injected with 150 units of PTE 1 day prior to sacrifice. The animal had been given a single injection of glycine-H^3 at 6 days of age. A thin layer of woven bone, stained heavily with periodic acid–Schiff, is present centrally. The matrix of the woven bone is not labeled, indicating that it had been formed prior to the injection of glycine-H^3. Within this region, however, the walls of osteocyte lacunae are reactive. Reaction bands (arrows) are present at the junction of the woven bone and the thick layers of lamellar bone above and below. These heavily labeled lamellae were formed within the first 4 hours after injection of glycine-H^3. The remainder of the lamellar bone (which is weakly and diffusely labeled) was formed subsequent to the injection. Accelerated resorption within the diploe is unrelated to the age or histologic organization of the matrix. Note that the ectocranial, but not the endocranial, surface (below) shows an increased affinity for the PAS stain. Autoradiogram, PAS-hematoxylin. (× 320.)

BIOLOGICAL LAB

State of Matrix in Sites of Preferential Resorption in Normal Rats

No relationship was apparent between sites of preferential resorption and the age, degree of calcification, amount of organic mass, histological organization, or chemical variations (differential staining) of the calcified matrices of bone and cartilage. For example, although no differences could be detected among the calcified cartilage intercellular partitions in regard to these variables, some of these partitions are resorbed in the zone of cartilage invasion, while others are spared. At the distal extremities of the metaphyseal trabeculae, resorption simultaneously removes the calcified cartilage cores and the thin osseous coating, despite differences in the age (the cartilage is slightly older than the bone), histological organization, organic mass content, and relative amounts of matrical constituents (Figs. 3, 8, and 9).* Similarly, in the skull, resorption of the walls of the diploic spaces involves removal of both woven and lamellar bone, without regard to their age (the woven bone usually is older: Young, 1962*b*) or any of the other variables studied, including degree of calcification (Figs. 4, 5, 11, and 13).

Sites of Preferential Resorption in Rats Treated with Parathyroid Extract

Bone resorption was markedly accelerated in all animals which received parathyroid extract, as indicated by the regional disappearance of calcified matrices in PTE-injected animals, compared with untreated controls. The greatest resorption occurred in regions which in uninjected animals could be identified as sites of normal resorption. In addition, resorption tended to spread from normally resorptive zones into adjacent areas.

For example, an increase of resorption in the zone of cartilage invasion resulted in the occasional partial or apparently complete detachment of metaphyseal trabeculae from the calcified intercellular partitions of the growth cartilage. At the distal end of the metaphyseal spongiosa, resorption was also accelerated, noticeably

* In some species significant differences in calcification occur as well (Owen *et al.*, 1955).

shortening the trabeculae (Fig. 19). Furthermore, resorption extended into the mid-trabecular region, normally characterized by osseous apposition. In extreme cases, the metaphyseal trabeculae were entirely resorbed. Similarly, an acceleration of subperiosteal resorption in the metaphyseal region of both ribs and tibiae resulted in the thinning of bone at this site. Not infrequently, the shaft in this region was perforated or fractured (Fig. 19).

In the skull, resorptive sites were related to the age of the animal, as in controls. In young animals (less than 10 days old), an increase in resorption (and in osteoclasts, stained supravitally with neutral red) was most marked endocranially, particularly near the bone margins. Ectocranial resorption near the bone centers also appeared to be increased. In older rats, in which development of the diploe had been initiated, the diploic spaces were noticeably widened in hyperparathyroid animals (Figs. 10 and 22). Surface resorption continued to predominate endocranially (Figs. 20 and 21), but was less prominent than in younger animals.

State of Matrix in Sites of Preferential Resorption in Rats Treated with Parathyroid Extract

In hyperparathyroid rats, as in normal animals, there was no relation between sites of selective resorption and any of the several variable features of the calcified matrices which were studied. This finding was typified by the prominent resorption within the diploe of hyperparathyroid rats more than 10 days old. The accelerated removal of bone was characterized by a progressive enlargement of the diploic spaces, without regard to whether the involved matrix was a "younger" region of lamellar bone, or an older region of woven bone with slightly less organic mass, slightly more basophilic material, appreciably more irregularly arranged collagen, slightly greater impregnation with mineral salts, and larger, more closely packed and irregularly arranged osteocytes (Figs. 10 and 22). In some of the older rats, woven bone which was resorbed in this process had been formed several weeks earlier (Fig. 22), whereas that simultaneously removed from the metaphyseal spongiosa was only a few hours old.

The Cellular Component in Resorption

The presence of osteoclasts was a characteristic, but not inevitable, accompaniment to demonstrable resorption. In some cases, osteoprogenitor cells (but never osteoblasts) were present in such regions. There was no evidence of decalcification (in agreement with Sognnaes, 1959), changes in organic mass (in contrast to Greulich, 1961; Takuma, 1962), or modifications in staining properties of the organic matrix beneath osteoclasts. Occasionally, bone surfaces with increased affinity for the PAS reagent were observed in animals treated with parathyroid extract (Heller-Steinberg, 1951; Gaillard, 1955*a*, 1955*b*; Laskin and Engel, 1956; cf. Kroon, 1958), but this could not be correlated with demonstrable resorption (Fig. 22). Frequently, a single osteoclast could be observed apparently engaged in resorbing a segment of matrix containing components of varying age, degree of calcification, histological organization, etc. (Figs. 3, 7, 9, 11, and 21). No cytoplasmic ingestion of calcium salts by osteoclasts was detected, nor was glycine-H^3 concentrated by these cells, even when they were present on heavily labeled bone surfaces (cf. Arnold and Jee, 1957).

Osteoclasts were generally characterized by large, pale-staining nuclei, prominent, RNA-containing (pyroninophilic, RNase-digestible) nucleoli of high organic mass (Fig. 7), and variable but appreciable cytoplasmic RNA. In normal animals (seldom in hyperparathyroid rats) some osteoclasts were seen with scanty cytoplasm and small, dark-staining nuclei, with insignificant nucleoli. Metachromatic (toluidine blue) granules and numerous small, PAS-positive granules (retained after incubation in saliva) were observed within the cytoplasm of osteoclasts. Nucleated inclusions, contained within cytoplasmic vacuoles, were a prominent feature of osteoclasts in hyperparathyroid animals, but were rare in controls. The included nuclei generally had little associated cytoplasm, and were small, dark-staining or pyknotic, of high organic mass, and characteristically lobulated (Figs. 15 to 17).

There is considerable variability in the size, shape, and arrangement of osteocytes in normal bone (Figs. 1, 2, 11, and 18). In regions of resorption, no unusual modifications in osteocytes or their sur-

roundings were observed. Occasionally, small groups of apparently enlarged osteocytes, well removed from bone surfaces, were seen in animals treated with massive doses of PTE. This was an infrequent rather than a common finding. In these hyperparathyroid rats, however, osteocytes were uniformly depleted of their normal stores of cytoplasmic glycogen (saliva-digestible, PAS-positive material). Chondrocytic glycogen was not noticeably decreased in these animals.

In regions of osteogenesis in normal animals, newly formed osteocytes near the bone surface were characterized by large size, abundant cytoplasmic and nucleolar RNA, large, pale-staining nuclei, absence of glycogen, and active synthesis of bone matrix (cf. Young, 1962*b*, 1963*a*). A surface layer of osteoid was generally discernible in such regions (Fig. 14). Osteogenic surfaces were inevitably coated with osteoblasts, and were characterized by a granular appearance of the matrix in preparations stained by Bodian's silver technique (Fig. 18). Within this new matrix, the walls of osteocyte lacunae and canaliculi were not stained. Slightly farther from the surface, and within all other regions of the bone, they were clearly delineated (Figs. 1 and 18).

In animals given large doses of parathyroid extract beginning 24 hours before sacrifice, no osteogenic surfaces, osteoid, or newly formed osteocytes were detected, owing to the rapid cessation of bone formation. During this process, osteoblasts became depleted of RNA, lost their ability to synthesize bone matrix (cf. Vaes and Nichols, 1962), and reverted to the osteoprogenitor state (Young, 1963*a*). Osteoprogenitor cells were characterized by a propensity to fuse in resorptive areas, forming increased numbers of osteoclasts, and by regionally accentuated cell proliferation, which led to a significant thickening of the endosteum and of the cellular lining of the diploic spaces. This proliferative response was associated with the production of a network of argyrophilic fibers (cf. Kroon, 1958).

Discussion

The techniques used in this study have made it possible to distinguish a large number of variable features within the mineralized

matrices of bone and calcified cartilage. In no case was there evidence that any of these features was effective either in determining regions in which resorption occurred, or in modifying rates of resorption within these regions. The possible resistance of osteoid to resorption could not be studied, since in normal animals uncalcified matrix was uniformly present in sites of osteogenesis, and in PTE-treated animals no osteoid was detected at the intervals examined.

The abnormally pronounced resorption occasioned by administration of parathyroid extract appeared to represent an exaggeration of normal resorption already in progress (cf. Engfeldt and Zetterström, 1954) and a spreading of this process into adjacent regions. This conclusion is consistent with experiments which have demonstrated that there is a lag between the time of uptake of bone-seeking isotopes and the time at which they are released from bone following parathyroid stimulation. At the time of administration, these osseous precursors are incorporated mainly in sites of osteogenesis (e.g., in the metaphyseal zone of bone apposition). Later, some of the labeled sites are reached by resorptive processes (e.g., in the metaphyseal zone of trabecular resorption). The lag between initial uptake and subsequent normal resorption is influenced by the age (rate of growth) of the animal. In 6-day-old rats, for example, regions of apposition in the metaphysis (labeled by matrix precursors such as glycine-H^3) may be found undergoing resorption as early as 4 to 6 hours later (Young, unpublished material). In older animals, this interval is progressively and appreciably lengthened. The metaphyseal zone of long bones represents a major site of resorption following parathyroid stimulation. Consequently, raised parathyroid hormone levels will accelerate the release of bone-seeking isotopes within hours after their administration in young animals, but will not stimulate their removal until later in older animals.

Thus, findings derived from experiments of this nature using young animals (interpreted as showing an effect on new bone) and those from old animals (interpreted as showing an effect on old bone) may be brought into harmony under the explanation offered above, that the hormone accelerates resorption in areas already characterized by resorptive processes. An acceleration of normal resorption

is also indicated by the finding that fluctuations in calcium levels in tissue fluids may occur within minutes after changes in parathyroid secretion in nephrectomized animals (Talmage, 1956, 1962). This suggests an immediate effect on the rate of resorption already in progress.

Since it is becoming increasingly apparent that the several types of bone cells represent different functional states of the same cell (Bloom *et al.*, 1958; Young, 1962*a*, 1963*a*, 1963*b*), and, further, that these cellular specializations are reversible, it is evident that all bone cells contain the genetic potential for bone resorption. In which of these functional specializations is this potential realized?

There appears no longer to be any doubt that *osteoclasts* are capable of resorbing bone and calcified cartilage (as well as dentin, cementum, and enamel: Sognnaes, 1959). Some reports claim, however, that osteoclasts may appear after resorption has taken place (Burrows, 1938; Gaillard, 1955*a*, 1955*b*), or may not appear at all, despite recognizable resorption (Engel, 1952; Storey, 1957; Urist and Deutsch, 1960). Apparently, groups of bone cells in which the resorptive response has been activated tend to coalesce, creating a multinucleated cell, yet need not necessarily do so in order to carry out this function. The ability of osteoclasts to phagocytize whole cells (indicated by the presence of nucleated inclusions within cytoplasmic vacuoles: cf. Arey, 1917; Jaffe, 1933) has been noted. This suggests the presence of a variety of lytic enzymes in these cells.

Many workers have brought forward evidence which suggests that *osteocytes* may be capable of limited resorption of their lacunar walls (Jaffe, 1933; Kind, 1951; Heller-Steinberg, 1951; Lipp, 1956; Kroon, 1958; Frost *et al.*, 1960). In the present material, however, no consistent alteration in osteocytes or their surroundings suggesting a significant activation of the resorptive potential was observed, even following massive doses of parathyroid extract. The loss of glycogen by osteocytes under such conditions (Heller-Steinberg, 1951; Laskin and Engel, 1956) probably represents a direct response to the hormone, since no glycogen depletion occurred in chondrocytes.

It has occasionally been stated (e.g., Follis, 1952) that *osteoblasts*

are capable of resorbing bone. In the present material, however, cells characterized morphologically as osteoblasts uniformly were engaged in formation (rather than destruction) of bone matrix (cf. Young, 1962*b*, 1963*a*). The same may be said of immature osteocytes, which retain osteoblast characteristics for some time after being trapped in bone. Furthermore, osteoblasts were sparse or absent in PTE-injected rats in which resorption was increased.

Endothelial cells have been implicated in resorption (Jaffe, 1933; Cameron, 1961). Others have suggested that *histocytes* are capable of resorption (Muhlethaler, 1953; Goldhaber, 1961) or can serve as precursors of osteoclasts (Hancox, 1956; Jee and Nolan, 1962). Conceivably, these cells (and others) represent mesenchymal specializations which have retained the capacity for resorption, and are capable of activating it under suitable circumstances.

A number of stimuli are known which appear capable of evoking the resorptive potential in bone cells. For example, resorption often occurs when bone is relieved of its normal, functional stresses (Geiser and Trueta, 1958), or when its component cells have been injured or killed. Pressure on bone generally leads to its resorption (Reitan, 1951; Storey, 1955; Young, 1959). Parathyroid hormone, cortisone (Storey, 1957; Urist and Deutsch, 1960), changing levels of sex hormones (in birds: Bloom *et al.*, 1958), and possibly vitamin A (Barnicot and Datta, 1956) all accelerate resorption. Diploic resorption may occur in response to proliferation of associated hemopoietic tissue (Ascenzi and Marinozzi, 1958). In tissue culture, resorption is stimulated by high levels of oxygen (Goldhaber, 1961).

Evidently, a wide variety of stimuli is capable of provoking a similar response from the cells of bone. This is a strong indication that the complexity lies in the reacting system, the cells themselves. The cellular response involves an integrated mobilization of metabolic machinery, which apparently precludes the simultaneous utilization of these resources for other specialized functions (such as bone formation).

Elicitation of the resorptive response probably involves the coordinated activation of a group of interrelated genetic loci, which code information required for the synthesis of the specific proteins (including enzymes) involved in resorption. Conceivably, the vari-

ous resorptive stimuli might act through "regulator" genes, which, by controlling the activity of several functionally related "structural" genes, could facilitate the evocation of integrated, complex cellular responses by relatively simple *microenvironmental* modifications (Young, 1963*b*).

By microenvironment is implied the entire complex of physical and chemical factors which impinge upon an individual cell. The functional specialization of individual bone cells is largely determined by the microenvironment in which they are situated (Bassett, 1962; Young, 1963*a*, 1963*b*). The central region of the metaphyseal trabeculae, for example, represents a microenvironment which elicits activation of the osteogenic complex, whereas conditions at the distal extremity of these trabeculae predispose the affected cells to resorption. At the overlapping edges of these microenvironmental "fields," intermediate cell types are found. These are apparently osteoblasts which are "de-specializing," as they come under the influence of the advancing resorptive field (cf. Young, 1962*a*).

Bone as a tissue provides a complex system of multitudinous, graded microenvironments. A systemic stimulus to resorption, such as parathyroid hormone, probably accentuates the tendency of these varied microenvironments to elicit the resorptive capacities of the involved cells. The initial response to raised hormone levels would presumably be an increase in resorptive rate by cells already engaged in resorption. Prolongation or accentuation of this stimulus would lead to an activation of the resorptive complex in cells at the periphery of existing resorptive fields. Such systemic resorptive stimuli would also modify normally osteogenic microenvironmental fields, slowing bone formation, and in extreme cases ultimately leading to its cessation.

Summary

Analysis of bone and calcified cartilage by a variety of techniques has made possible the identification of calcified matrices varying in age, degree of calcification, histological organization, and chemical composition. None of these variable features proved to be critical in determining sites of preferential resorption in normal young rats,

or in rats in which resorption had been stimulated by the administration of parathyroid extract. In the latter group, resorption appeared to represent an acceleration of resorption already in progress, and an extension of this process into adjacent areas. The role of the cellular microenvironment in eliciting the resorptive capacities of bone cells has been emphasized. The cellular response to appropriate microenvironmental modifications apparently involves the complex, coordinated activation of integrated portions of the cell's total genetic information, resulting in the onset of resorption, which then proceeds without regard to structural variations in the resorbable tissue.

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18

Structure-Function Relationships in the Osteoclast

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IT WOULD nowadays be generally accepted that the osteoclast is actively involved in some way in the resorption of bone (Hancox, 1963), but its exact role and mode of action remain very obscure. The results to be described below bear on this problem. They come partly from morphological studies with the electron microscope, and partly from cytochemical work at the light microscope level.

Materials and Methods

The electron micrographs were obtained from embryonic fowl bone fixed in Palade's buffered osmium tetroxide. Araldite was used for embedding, and the results were much superior to those we had obtained in earlier work (Hancox and Boothroyd, 1961) with methacrylate.

The tissue cultures to be described were simple 24- to 48-hour hanging-drop preparations (fowl plasma and embryo extract) of embryonic fowl bone. Osteoclasts wander out from the bone into the surrounding plasma (Hancox, 1946). The cultures were prepared by Miss S. Warner, who also performed the cytochemical tests.

Results

Some of the main topographical features of a typical embryonic avian osteoclast as they would appear in a low-power electron micrograph are shown in diagrammatic form in Fig. 1. Several zones along the line where the cytoplasm adjoins the bone can be differentiated

Fig. 1. Diagrammatic representation of the main features seen in osteoclasts with low-power electron microscopy. Bone matrix represented as black, above; osteoclast below. Seven nuclei (*N*) are shown. The rectangles lettered A, B, and C refer to zones discussed in detail in the text; zone A is the site of the traditional "brush" or "striated" border. *P*, pinosome; *M*, mitochondrion; *V*, cytoplasmic vesicles.

on the basis of structural differences. Three of these will be discussed below; their main characteristics are as follows.

Zone C

The cytoplasm overlying the matrix is usually poor in organelles such as ribosomes, rough or smooth-walled sacs and vesicles, mitochondria, etc.; it has a finely granular amorphous appearance (Figs. 2, 3, 4, and 6). The cell membrane follows the contour of the bone edge closely except that here and there it seems to form short, blunt channels leading inward (Fig. 3). Loose or detached bone salt crystals can sometimes be distinguished within these (Fig. 6).

Appearances suggest that cytoplasm in this area was neither elaborating any secretion nor pouring it forth onto the bone surface at the moment the cell was fixed. It is interesting that Dudley and Spiro (1961, Fig. 13) obtained a similar cytoplasmic picture in what they describe as "endosteal lining cells," which they regard as forming a more or less inert covering membrane.

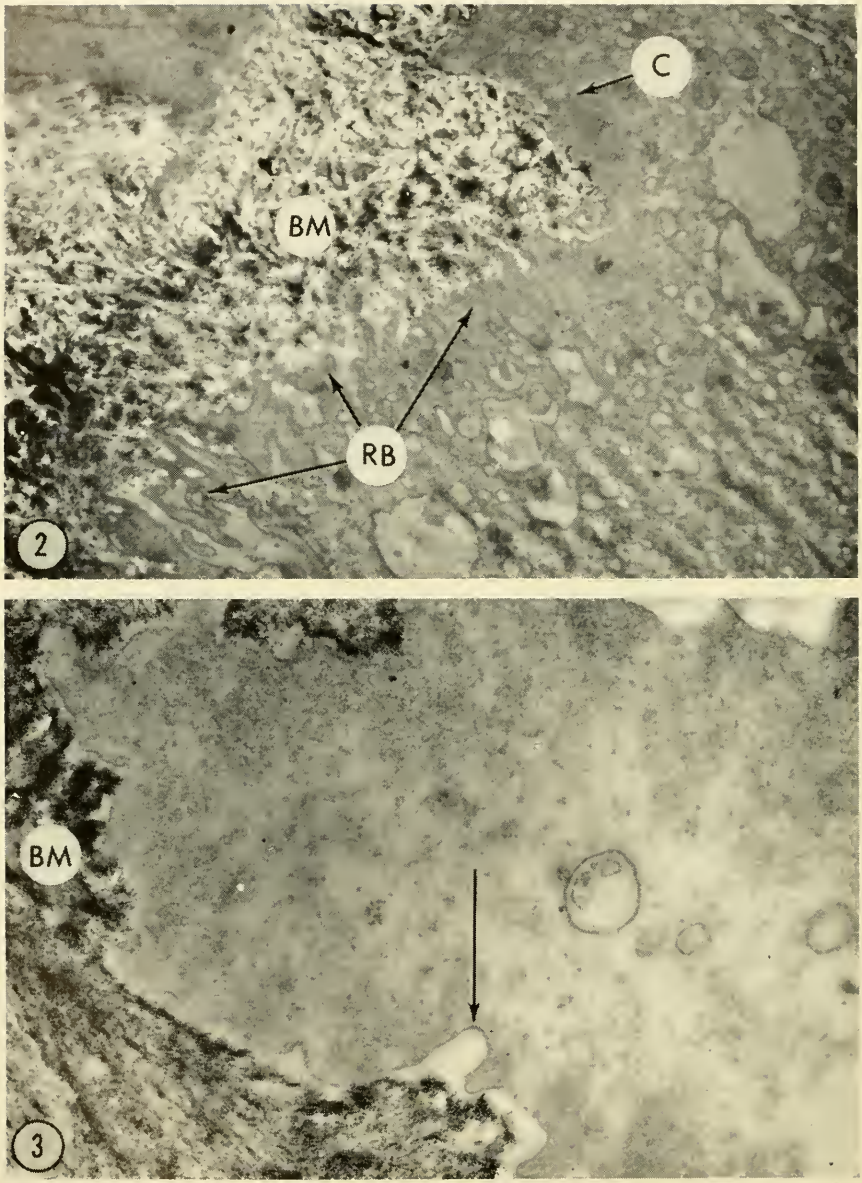
Zone B

This constitutes a transition zone whose length is very variable; it may be practically absent. The chief characteristics are the presence, first, of cytoplasmic vesicles gathered near the cell membrane, and, second, of more numerous and longer channels leading in from the surface. Bone salt crystals can be recognized in these, and they have obviously traveled some distance from the matrix (Fig. 4).

Zone A

This, of course, is the "brush" or "striated" border area of light microscopy (Kölliker, 1873; Kroon, 1954). Scott and Pease (1956) seem to have been the first to describe its ultrastructure. They thought it similar to the folded membrane of macrophages described by Palade (1955) and called it the "ruffled" border; this name is generally employed now in electron microscopy.

In our material the border consists of a complex system of cytoplasmic folds and projections separated by cleftlike spaces of variable appearance, and an associated system of cytoplasmic vacuoles and vesicles. At one end of the scale are relatively fine canals, often



FIGURES 2 AND 3

branching, which can sometimes be seen to terminate in saclike vesicles bounded by a single membrane (Figs. 7 and 9). Generally there are multitudes of other vesicles, structurally identical, in the near-by cytoplasm. At the other end of the scale (Figs. 2, 5, 7, 8, and 10) are broader channels leading into large vacuoles, probably pinosomes (Hancox and Boothroyd, 1961).

Bone salt crystals can be recognized in both the finer and the broader channels and also in the more distal pinosomes. Their appearance in such places has been described in most of the published work on the electron microscopy of osteoclasts (Scott and Pease, 1956; Gonzales and Karnovsky, 1961; Hancox and Boothroyd, 1961; Dudley and Spiro, 1961; Cameron and Robinson, 1958).

The occurrence of base collagen fibrils between folds of the ruffled border does not seem to have been encountered by other workers. Collagen occurs constantly, however, in our material (Figs. 5, 7, 10, 11, and 12). The fibrils seem to protrude from the surface of the calcified matrix and are closely invested by ruffled border folds. The interfold spaces continue outward into the cytoplasm in fine channels which may branch before ending in vesicular sacs. More rarely, collagen cross-banding can be identified in the larger and more distal pinosomes (Fig. 10). Partly demineralized fibrils are also seen.

Discussion

Turning next to the possible functional implications of these structural findings, two points particularly seem worth discussing, namely, the presence of free or detached bone salt crystals and the occurrence of collagen fibrils apparently denuded of their mineral.

Figures 2 to 12 are electron micrographs made from unstained sections of osmium-fixed bone embedded in Araldite.

Fig. 2. Low-power electron micrograph of part of an osteoclast, at right, in contact with a trabeculum of bone labeled *BM*. The position of zone C is indicated by that letter, and the arrow points to the amorphous cytoplasm typical of this zone, which is seen at higher magnification in Figs. 3 and 6. The position of the ruffled border (*RB*) of zone A is arrowed. (× 8000.)

Fig. 3. Bone matrix (*BM*) at left; apposed to it is the amorphous, inert-looking cytoplasm typical of zone C. It contains a few ribosomes and vesicles. A short, blunt cytoplasmic channel is arrowed. (× 45,000.)

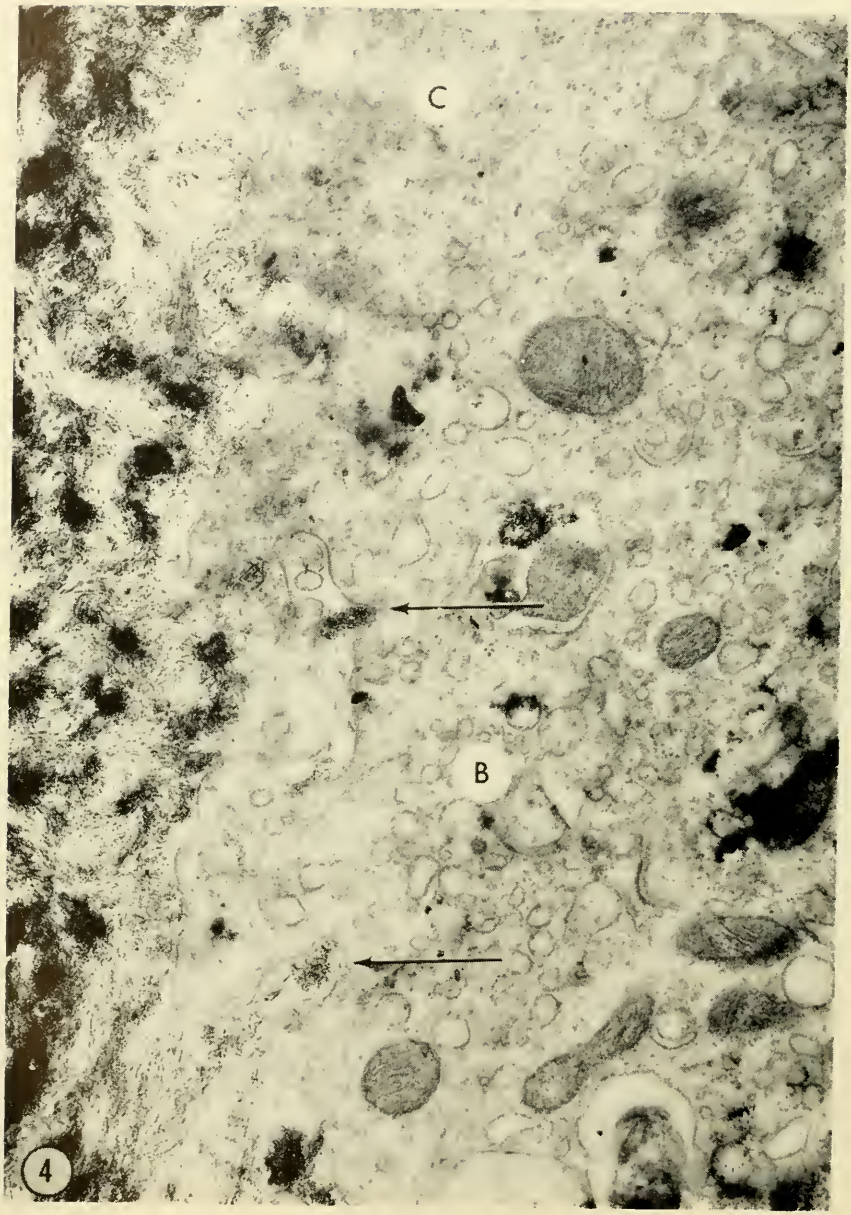


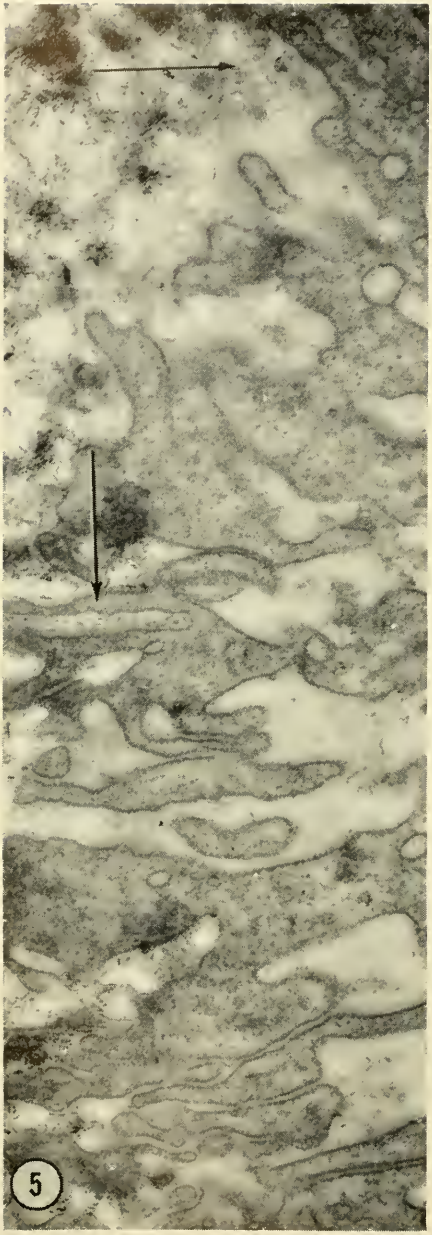
FIGURE 4

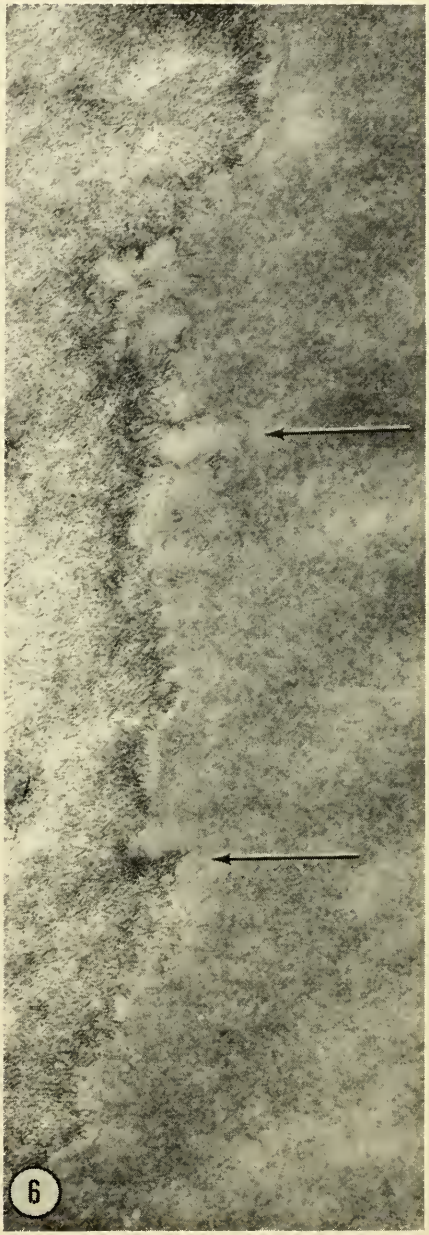
Loose, detached crystals have been described within cytoplasmic channels in the ruffled border and its vacuoles in all previous published reports on the electron microscopy of the osteoclast (Scott and Pease, 1956; Cameron and Robinson, 1958; Gonzales and Karnovsky, 1961; Hancox and Boothroyd, 1961; Dudley and Spiro, 1961). Their strict confinement to these sites and their constant occurrence seem to rule out any question of mechanical shift by the knife edge during sectioning, and one can conclude that loose crystals genuinely occur in such areas as zones A and B.

Concerning zone C, however, the question of artifact has to be considered carefully. Here again, however, artifact seems unlikely; the tissue samples, of course, were supported by and permeated with hard plastic, and if the crystals had been translocated by the knife edge one would expect to find telltale scars or tears in the Araldite, but these are not seen. In zone C, free crystals are encountered below what seems to be inert cytoplasm. It is very interesting that similar loose crystals can be distinguished in Dudley and Spiro's Fig. 13 (1961), where they are present below the inert-looking cytoplasm of an "endosteal lining cell." This observation suggests that crystals can be detached from the surface of normal bone, or, rather, that the osteoclast is not required for the detachment of crystals, and of course it raises the whole question of the nature of the forces which normally bind crystals to collagen, and what is normal bone.

Passing from zone C through B to A, more crystals can be seen to have been shifted and over longer distances. How the crystals are detached is uncertain. Other workers, of course, have proposed that the primary change is solution of the collagen; if it is removed first, it would, of course, leave the crystals free. This view of the sequence of events is based chiefly on failure to detect demineralized collagen fibrils in the subjacent matrix (Scott and Pease, 1956; Gonzales and Karnovsky, 1961; Dudley and Spiro, 1961). However, both with Araldite in the present work and with methacrylate in our previous

FIG. 4. Bone matrix runs vertically down the left margin, and osteoclast cytoplasm occupies rest of field. At top, zone C is indicated. There is a transition to zone B features below; arrows indicate its short cytoplasmic channels, containing bone salt crystals. Note innumerable cytoplasmic vesicles and mitochondria. (× 35,000.)





FIGURES 5 AND 6

study (Hancox and Boothroyd, 1961), collagen fibrils completely or partially (Fig. 5) denuded of their apatite crystals occur constantly. This strongly suggests that the primary change is the removal of crystals, collagen disappearing subsequently. The reasons for this interesting discrepancy are not clear. Naturally the question of artifact has to be reviewed carefully with respect to the possibility of loss of crystals during specimen preparation or in the electron beam. It is unlikely, however, that they would be removed from collagen in this way while persisting in near-by channels and vacuoles. All the same, this point merits further study, and Boothroyd is currently working on it. Another possibility is that a species difference is involved. Scott and Pease (1956) worked with kitten tissue; Gonzales and Karnovsky (1961) used rat tissue; Dudley and Spiro (1961) studied material from man and 10-week-old chickens, but give no indication in their paper of the species of the osteoclasts illustrated.

If crystals indeed leave the fibril first, then presumably the forces which anchor them must be weakened or abolished. What the forces are, and how they can be affected, remains obscure. It is difficult to see how acid decalcification (Kölliker, 1873) or chelation (McLean and Urist, 1955) can be involved; these should lead to disappearance of the crystals rather than their shift from matrix to cell.

At all events, it would follow that as demineralization proceeds, the underlying fibrils will be exposed. Those which protrude from the resorbing edge seem to become gathered up by folds in the ruffled border, and their fate, presumably, is to be digested away by enzyme action as erosion of the matrix deepens.

No solid formed bodies are seen in the osteoclast cytoplasm which might be the carriers of enzymes. Dense bodies, such as have been

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FIG. 5. Edge of bone matrix runs vertically downward just beyond left-hand margin. The field is occupied by ruffled border of zone A. Channels, some wide and pinocytotic, and others finer, lead (horizontally) from matrix into cytoplasm. A demineralized collagen fibril (horizontal arrow) can be seen toward top; another (vertical arrow) still appears to have a few crystals attached. (× 30,000.)

FIG. 6. Zone C. Bone matrix runs vertically down left side of figure. The typical "inert-looking" cytoplasm fills rest of field. There are several short, blunt cytoplasmic channels (arrows) apparently containing free bone salt crystals. (× 70,000.)

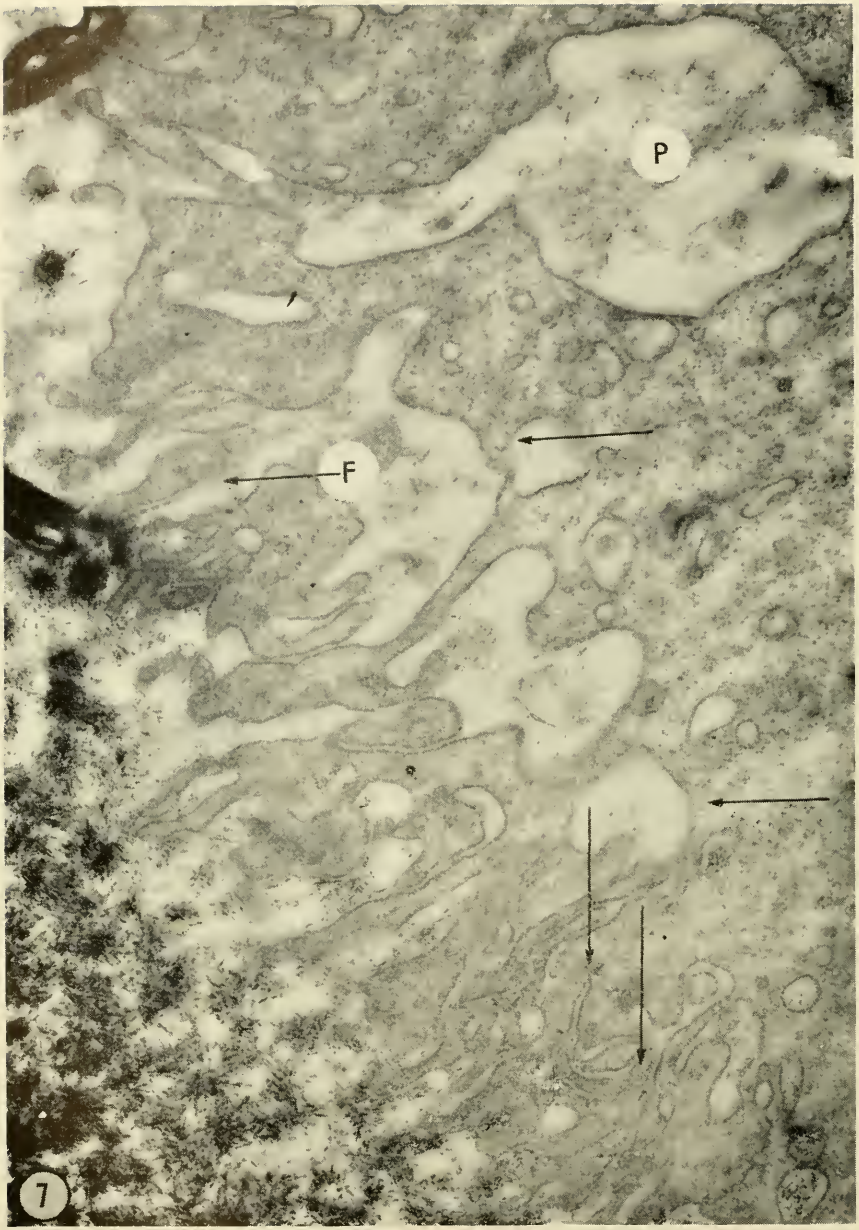

FIG. 7. Zone A, ruffled border. Bone matrix to left. Note pinosome (*P*) at top, and others in varying section plane (horizontal arrows). Finer channels (vertical arrows) also lead off from matrix edge. Demineralized collagen (*F*) can be seen between cytoplasmic folds. (× 70,000.)

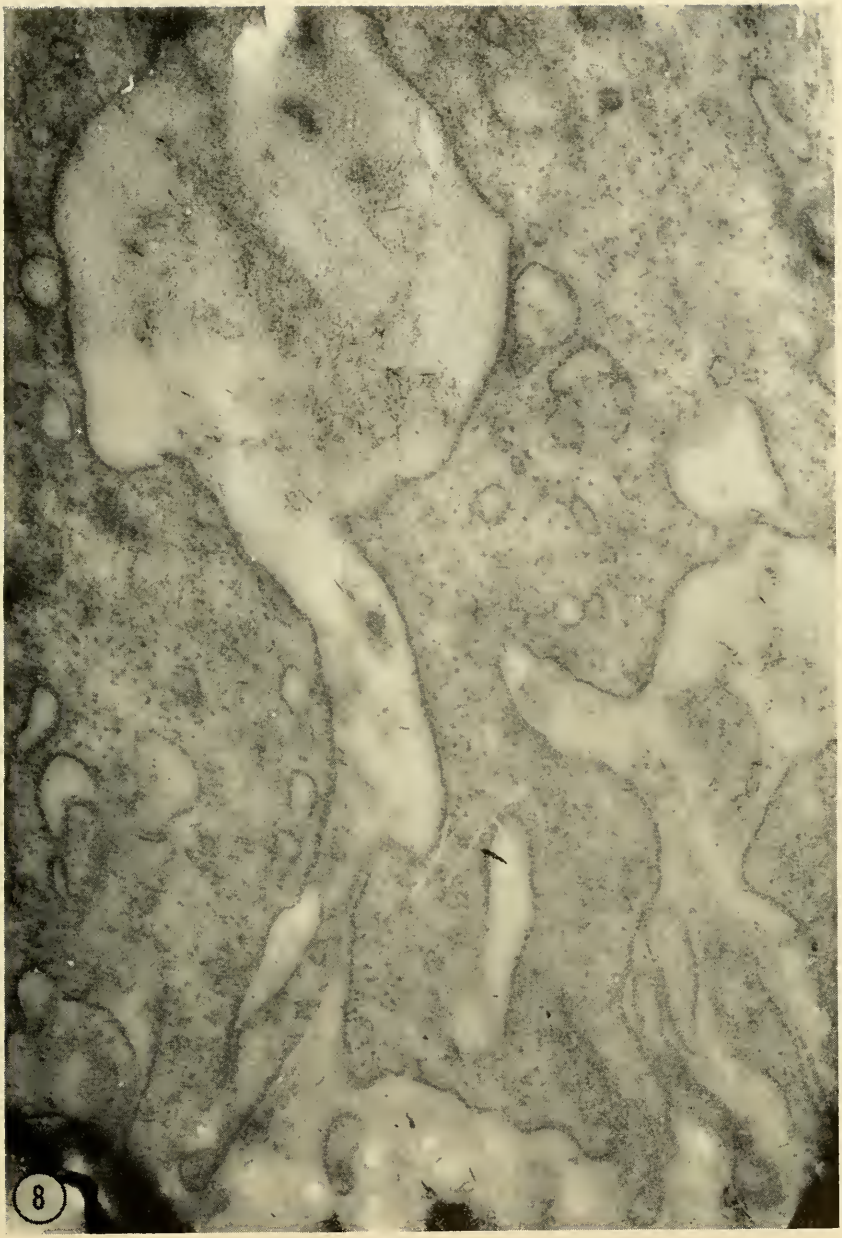

FIG. 8. Pinosomes from Fig. 5 enlarged to show their contained bone salt crystals. Note ribosomes and vesicles scattered in the cytoplasm. (× 45,000.)

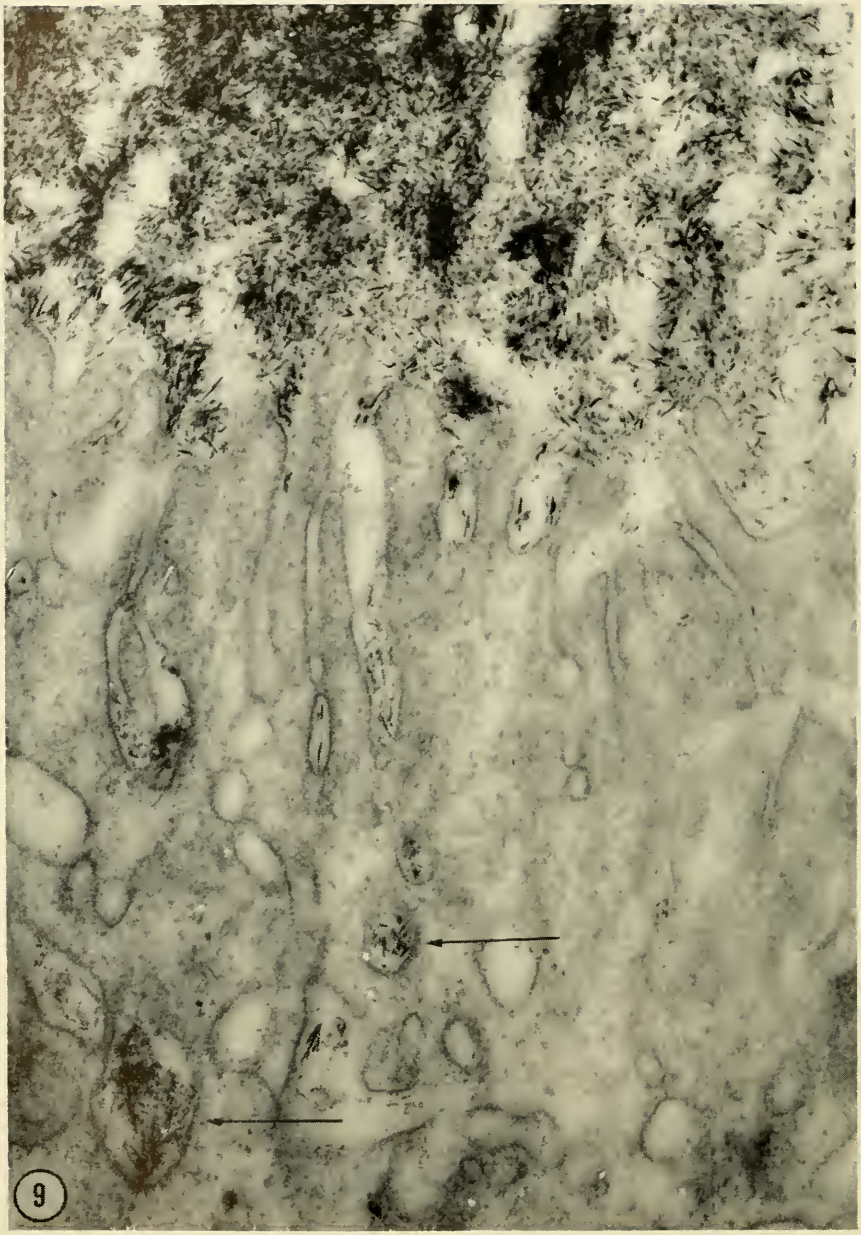

FIG. 9. Ruffled border of zone A. Fine channels lead down from bone above. Bone salt crystals can be seen within. Channels probably terminate in saclike vesicles (arrows); these also contain crystals. (× 50,000.)

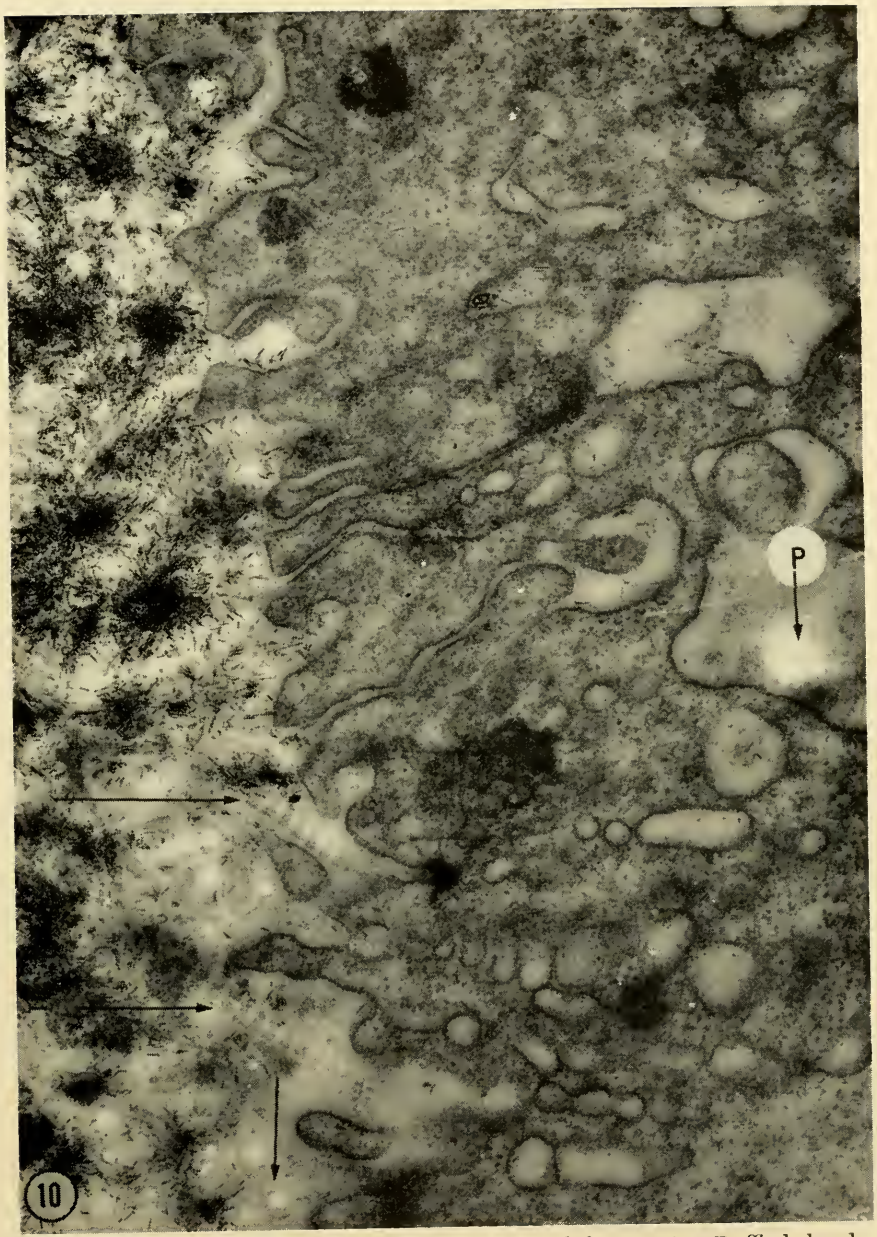

FIG. 10. Bone matrix runs vertically down left margin. Ruffled border adjoins. Collagen denuded of bone salt seen cut in longitudinal section (horizontal arrows) and, possibly, transversely (vertical arrow). A pinosome (*P*) is indicated. In original electron micrograph collagen cross-banding is discernible at arrows, but it is difficult to make out in positive print. (× 40,000.)

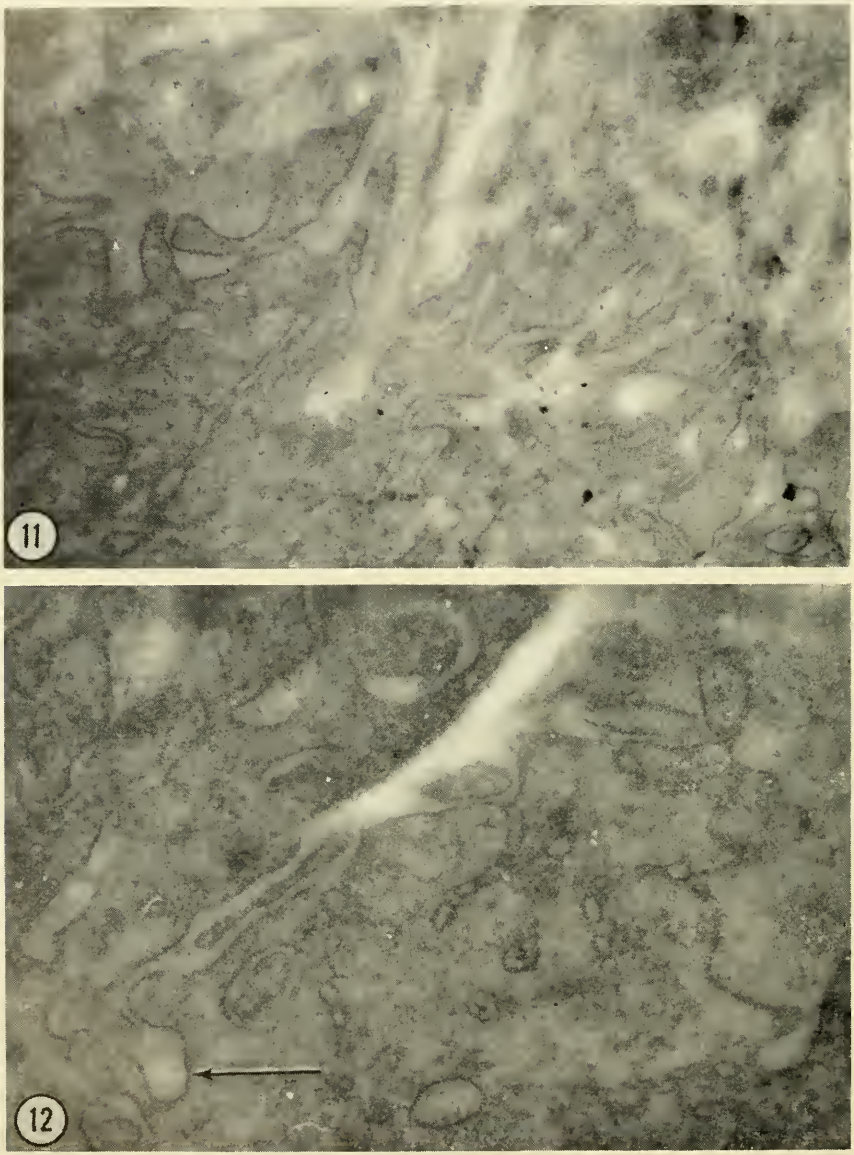

FIG. 11. Ruffled border folds enclosing collagen fibrils. (× 30,000.)
FIG. 12. Collagen fibril surrounded by ruffled border folds. Channels lead off from the fold and end, apparently, in a saclike vesicle (arrow). (× 35,000.)

identified with lysosomes, the carriers of hydrolytic enzymes in other cells (de Man *et al.*, 1960; Holt and Hicks, 1961), are not to be seen in the osteoclast. On the other hand, as the electron micrographs show, the cytoplasm of this cell is characteristically packed with small vesicles bounded by a single membrane. It is naturally tempting to think of these as the carriers of the enzyme cocktail needed for solubilization of the collagen and the ground substance. They might be a form of, or related to, "lysosomes," or "phagosomes" (de Duve, 1959; Novikoff, 1960), or "cytolysomes" (Novikoff and Essner, 1962). Perhaps they empty into the cytoplasmic folds, much as "dense bodies" believed to contain hydrolytic enzymes have been thought to coalesce with liver cell vacuoles (de Man *et al.*, 1960). Rose (1957), of course, has watched small dark cytoplasmic bodies moving toward and fusing with pinosomes in living cells and has proposed that this may represent enzyme transfer.

It is very unfortunate that little or nothing is known about the cytochemistry of the osteoclast at the electron microscope level. However, there is quite a lot of information from the light microscope, and osteoclasts are known to contain a wide range of enzymes. Leaving aside their oxidases, dehydrogenases (Walker, 1961; Burstone, 1960*b*), and carbonic anhydrase (Simasaki and Yagi, 1960), they possess acid phosphatase (Burstone, 1960*a*, 1960*c*; Changus, 1957), leucine aminopeptidase (Burstone, 1960*a*; Lipp, 1959), and α,β-glycosidase and galactosidase (Schlager, 1959, 1960). Such enzymes are often considered to be involved in the breakdown of soft tissue constituents elsewhere in the body (de Duve, 1959; Novikoff, 1960; Cabrini, 1961), and there seems no reason why they should not have a similar effect on bone matrix once decalcified.

Cytochemical observations made in our laboratory (Hancox and Warner, unpublished) on whole osteoclasts in tissue culture have provided some suggestive results. The advantage of these over sectioned cells is that cytological detail is very much better preserved. Thus far, acid phosphatase, leucine aminopeptidase, and β-glucuronidase have been studied. The reaction product is located in the cytoplasm in the form of droplets and granules whose size and number seem to coincide quite well with those of the larger vesicles seen

in electron micrographs. Obviously, the next step is to locate the reaction product in the electron microscope. A further point of interest is that the various osteoclasts present in a given culture differ in their content of a particular enzyme. Some are strongly positive, some weakly so, and others more or less negative. This perhaps might be another example of the existence of different degrees of "readiness" for phagocytosis as postulated by Holt and Hicks (1961).

Summary

Electron microscopy seems to indicate that bone salt crystals are somehow loosened from embryonic avian bone matrix and are swept up into channels and vacuoles in the osteoclast; the exposed collagen is enclosed in folds of the ruffled border in a way which strongly suggests a process of continuous digestion. Cytochemical work with the light microscope shows that lysosome-like hydrolytic enzymes occur in the cells. But much more information is needed, particularly about the shift of crystals and about the enzyme cytochemistry of the cell at the electron microscope level.

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19

Bone Destruction by Multinucleated Giant Cells

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THE osteoclastic ability of foreign body giant cells was noted by Bujard (1946), who injected particles of ground-up bone under the skin or into muscles and found them surrounded by giant cells which behaved like osteoclasts. A little later Ham and Gordon (1952) implanted dead autogenous bone into muscles and observed the formation of typical osteoclasts around the implants. They concluded that these cells did not need to arise from osteogenic cells or from osteoblasts. Bujard considered that these osteoclasts could arise from mesenchymatous cells that were in the "état histocytaire."

In the present experiments a similar technique was used in the investigation of several problems: (1) to determine whether these giant cells had the same cytochemical properties as osteoclasts found around bone in its normal position; (2) to study the effect of chemical or biological alterations of implanted bone upon the ability of these giant cells to resorb it; in the present paper, the reaction with rachitic osteoid is described; (3) to determine whether the osteoclasis of the devitalized bone implants was dependent on the parathyroid glands.

Methods

Rats. Young male animals of the Holtzman strain weighing 80 to 100 gm were used.

Operative technique. Under ether anesthesia, a piece of the scapula was removed aseptically. Part was preserved for histological examination (called the reference section) and the other part was treated in various ways and ultimately implanted into the same animal under the skin of the back, the implants thus being autogenous. At stated intervals the animals were sacrificed and the bone and surrounding tissue removed for examination. Some animals in each group were injected with trypan blue before the implant was removed, to detect the presence of phagocytic cells around the implant.

Treatment of bone implants. Decalcification was carried out with 0.5 M ethylenediamine tetraacetic acid at pH 7.0 for 7 to 10 days. The bone was well washed before implantation. Some of the reference bones that had been decalcified were ashed at 900°C to determine if decalcification had been complete. Devitalization was carried out by repeated freezing to −40°C and thawing. The presence of empty osteocyte lacunae was taken as evidence of devitalization. In no case was any fixative employed.

Histological methods. The following were used: hematoxylin and eosin; the von Kossa method for phosphate in calcified tissues, the sections being treated with thiosulfate before mounting; the Gomori (1933) method for calcified tissues.

Histoenzymologic methods. For the demonstration of enzymes, decalcified scapulae which had been implanted for 2 weeks were frozen in isopentane, cooled by a dry ice–acetone mixture. Sections 10 microns thick were cut on a cryostat, mounted on coverslips, and incubated at room temperature in the various substrates. The following procedures were used: for cytochrome oxidase, that of Burstone (1961*a*), using 8-hydroxy-1:4-naphthoquinone as a coupling agent; Burstone's method (1961*b*) for acid phosphatase, using naphthol AS-BI phosphate as substrate, and fast red violet LB

salt as the coupling agent; for succinic dehydrogenase, the method of Seligman and Rutenburg (1951); the method of Nachlas *et al.* (1958*a*) for diphosphopyridine nucleotide–linked dehydrogenases, malic, lactic, and glutamic; for DPN diaphorase, DPNH (0.5 mg/ml) was substituted as substrate in the procedure of Nachlas *et al.* (1958*a*); for the triphosphopyridine nucleotide–linked isocitric dehydrogenase, that of Nachlas *et al.* (1958*b*); and for TPN diaphorase demonstration, TPNH (0.5 mg/ml) was substituted as substrate in the last-mentioned procedure. In the demonstration of the above diaphorases and dehydrogenases, tetranitro blue tetrazolium was substituted for nitro-BT to minimize artifactitious staining on lipid-aqueous interfaces (Rosa and Tsou, 1961). Control sections were incubated in the absence of substrate, and in the demonstration of cytochrome oxidase were treated with 10^{-4} mole of NaCN prior to incubation. All incubations were for ½ hour except for acid phosphatase and isocitric dehydrogenase, which were for 1 hour. Maxillae and tibiae of 3-day-old rats were processed to compare the enzyme activities of skeletal osteoclasts with those of the giant cells about the implants.

Details of the Experimental Groups

Group 1. The scapula sample was removed, decalcified, and implanted 14 days later. Groups of 3 to 7 animals were killed at weekly intervals after implantation up to 8 weeks. In some groups, prior to implantation, the decalcified scapulae were blotted on filter paper and weighed to the nearest 0.1 mg, and after removal were similarly reweighed.

Group 2. Rats were put on the rachitogenic diet of Jephcott and Bacharach (1926) for 28 days. Portions of the scapula were removed and devitalized by repeated freezing and thawing but were not decalcified. The animals were in the meantime returned to a normal diet and 2 weeks later the bone was implanted. Groups of 4 to 17 animals were killed at weekly intervals up to 4 weeks later, when the implant was removed and sectioned.

Group 3. Animals were treated similarly to those of group 2, but the bone was decalcified and implanted 2 weeks after removal. Groups of 4 or 5 animals were killed 1 to 3 weeks later.

Group 4. Pieces of muscle or skin were removed from normal animals and treated with EDTA for the same length of time as the bones in group 1. After implantation, 4 animals were killed weekly up to 4 weeks later.

Group 5. Animals had bacterial agar hydrocolloid implanted under the skin. Four animals were killed weekly up to 6 weeks later.

Group 6. This part of the experiment is in a preliminary stage. Decalcified and devitalized bone implants were used. Parathyroid extract (100 units/100 gm body weight for 4 days) was administered to some of the animals before the implants were removed, and other animals were parathyroidectomized with a cautery just prior to bone implantation. Groups of 4 rats were killed at intervals up to 8 weeks.

Results

Group 1

Decalcified bone had been deliberately chosen for the study of the giant cells, since it was possible to cut the specimens without further decalcification and thus better preserve enzyme and morphological details. The morphology and number of giant cells were the same as was found around devitalized calcified bone. All implants were completely devitalized as judged by empty osteocyte lacunae. They were also completely decalcified before implantation, as shown by analyses of the reference sections. None of the implant sites became septic, and this applied to all the implants in all groups.

There was a strong small-celled reaction around each implant early in the experiment. This became less intense with time, and later a fibrous capsule surrounded the implant. After 1 week multinucleated cells could occasionally be seen around the bone, occurring specially at the sectioned ends. By 2 weeks many more were seen, and to judge from slide counts, the number was maximal at

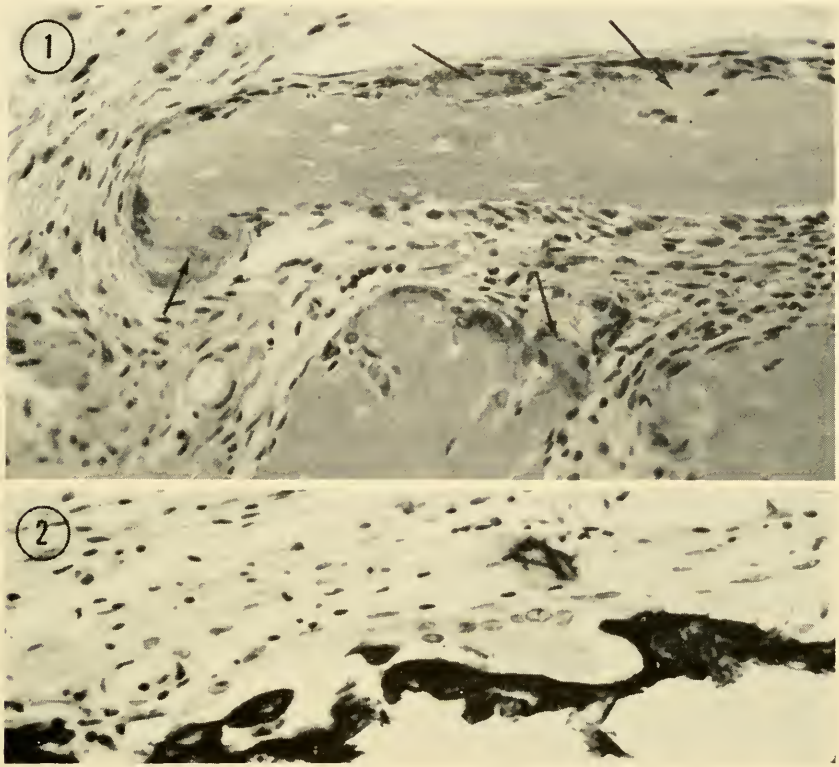


FIG. 1. Autogenous devitalized bone after 2 weeks of implantation. A number of giant cells (arrows) are seen especially toward the ends of the bones. Note associated cellular reaction. Hematoxylin and eosin. (× 350.)

FIG. 2. Autogenous bone decalcified before implantation which subsequently recalcified (black areas). Implant was removed after 3 weeks. Note giant cell in Howship's lacuna, and the nuclei on the periphery away from the bone. Von Kossa, hematoxylin and eosin. (× 350.)

about this time (Fig. 1). Some of the bones slowly recalcified after implantation, as could be seen in the von Kossa and Gomori sections. Before this happened, however, giant cells were seen in many places in contact with uncalcified surfaces. Later, as calcification became more extensive, these cells attacked uncalcified and calcified bone indiscriminately (Fig. 2). A number of macrophages which had engulfed trypan blue were seen around all the implants,

but at some distance from the bone. In no case had the giant cells engulfed trypan blue. Almost all the giant cells of diameters up to 100 microns had up to 12 nuclei arranged circumferentially around the cell, and very few had less than 6 nuclei. They were usually much larger than osteoclasts found around bone, in which the nuclei are usually more central. The cytoplasm was generally acidophilic, and all the cells were seen tightly applied to bone. The only other foreign bodies around which they were found were hairs which had accidentally been introduced. The giant cells were sometimes present in a long, thin shape, around projecting parts of bone, or else were in typical Howship's lacunae, where they were circular or oval in shape. No intermediate types with 2 or 4 nuclei were convincingly seen. This might be due to the timing procedure adopted, which failed to catch cells at this stage; but it seemed that the appearance of the multinucleated cells was a very sudden process.

Loss of weight of the implants was evident by 2 weeks, the time at which the giant cells began to appear in significant numbers. Thereafter it proceeded at a constant rate (Fig. 3).

The giant cells about the decalcified implants were exceedingly active for all the oxidative enzymes studied, the activity of these cells being equivalent in intensity to that of the osteoclasts of the skeleton of the newborn rat (Figs. 4 to 9). Acid phosphatase activity was less intense in the giant cells about the implant than that seen in the skeletal osteoclasts (Figs. 10 and 11). In some cases, the giant cells were flattened and difficult to differentiate from the surrounding cells (Fig. 7). When an enzyme process was applied, it became immediately obvious which they were (Fig. 8).

Group 2

The sections of devitalized bone from rachitic animals showed a different picture from those of group 1. The von Kossa and Gomori techniques showed that calcification of the rachitic osteoid began soon after implantation. By 1 week a few giant cells were seen on calcified surfaces, but they were never seen on osteoid till it calcified (Figs. 12 and 13). As the experiment proceeded, the bone became more heavily calcified and more giant cells were seen attacking it.

Group 3

This group of rachitic decalcified scapulae was a control on group 2 and was included to ensure that omitting decalcification was not responsible for the inability of the giant cells to attack osteoid. Giant cells appeared around the implants 2 weeks after implantation and were observed in increasing numbers with the passage of time. It was of course impossible to distinguish with any certainty rachitic osteoid from decalcified bone, but osteoclasis proceeded with the usual vigor.

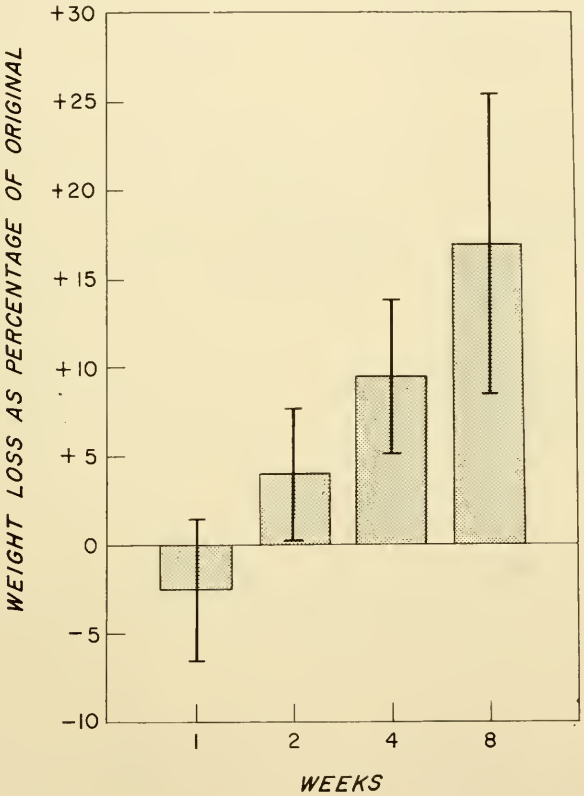
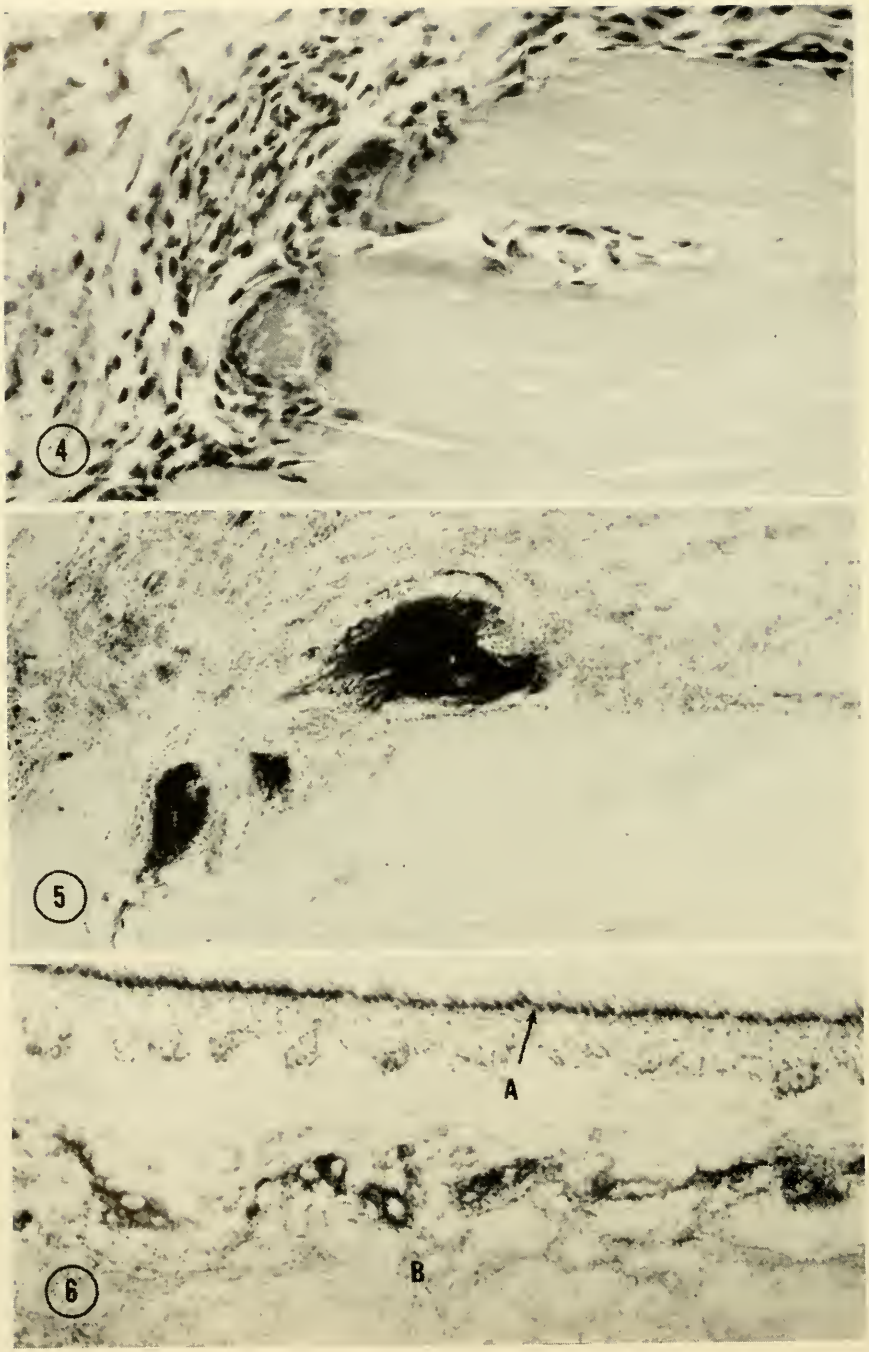


FIG. 3. The loss of wet weight of devitalized bone implants. There were 7 animals in each group. The bars show the means plus and minus the standard deviation.



FIGURES 4 TO 6

Groups 4 and 5

No giant cells were seen around dead muscle or skin, or around the foreign material introduced. The muscle was rapidly fragmented and was invaded by a number of macrophages. The skin appeared resistant to resorption and was walled off with connective tissue. The inert foreign material likewise was walled off with a thin layer of fibrous tissue, and no specific cell reaction was seen.

Group 6

Under the experimental conditions employed, no effect at all on the number or time of appearance of giant cells was caused by parathyroid extract or by parathyroidectomy. Administration of parathyroid extract caused a large increase in the number of osteoclasts on the tibia of the same animal, and parathyroidectomy was successful as judged by the marked hypocalcemia found in the animals after starvation for 24 hours.

Discussion

The first problem considered in this project was whether or not the giant multinucleated cells were comparable to osteoclasts.

Other workers have investigated the enzyme content of giant cells. Baker and Klapper (1961) implanted polyvinyl sponges subcutaneously into adult rats and carried out histochemical tests on the giant cells that appeared. They found succinic dehydrogenase, cytochrome *c* oxidase, diphosphopyridine nucleotide diaphorase, and lactic and malic dehydrogenases, the enzymes being present in

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Fig. 4. Giant cells are seen at the end of the decalcified, devitalized bone. Implant was removed after 2 weeks. Note the associated cellular reaction. For comparison with Fig. 5. Hematoxylin and eosin. (× 350.)

Fig. 5. Parallel section to Fig. 4 was reacted for succinic dehydrogenase. Note intense activity in giant cells, which are larger than the osteoclasts seen in Fig. 6. (× 350.)

Fig. 6. Labial alveolar bone of a 3-day-old rat, taken from around the upper incisor and reacted for succinic dehydrogenase. Note the positive reaction in osteoclasts resorbing bone (*B*). Positive reaction also seen in basal end of ameloblasts (*A*). (× 350.)

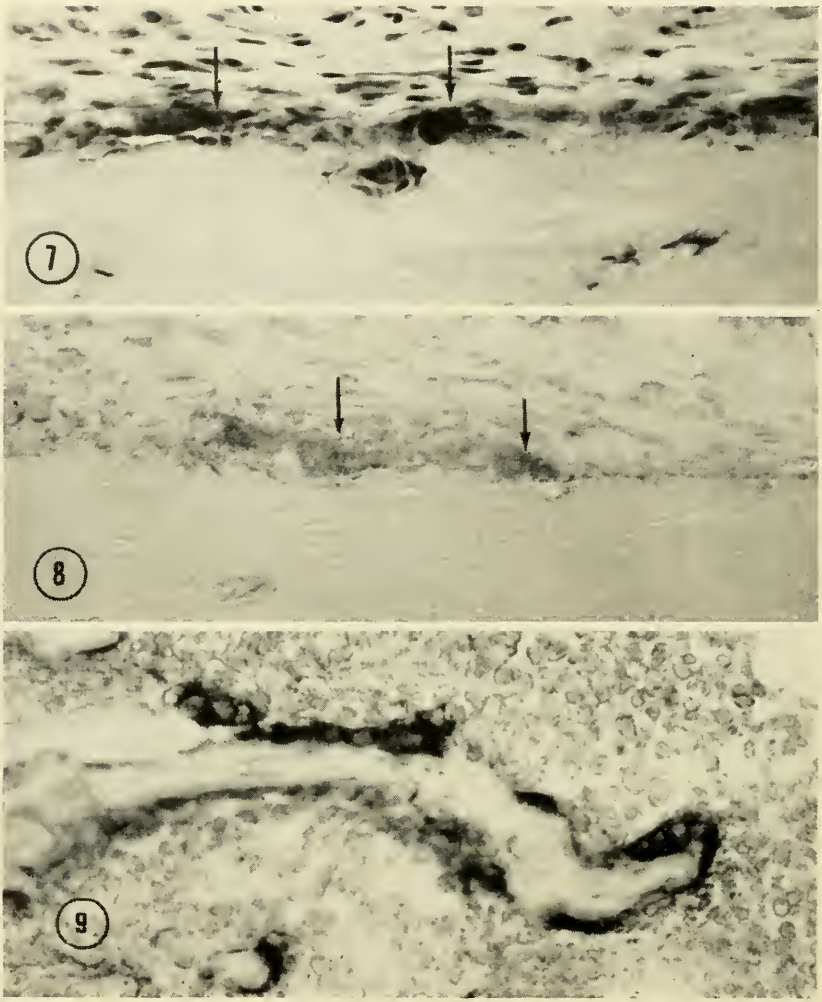

FIG. 7. Decalcified implant removed after 2 weeks, showing small multinucleated cells (arrows), possibly an early stage of giant cell development. Hematoxylin and eosin. (× 350.)

FIG. 8. Parallel section to Fig. 7 reacted for lactic dehydrogenase. Note activity in the early forms of giant cells (arrows). (× 350.)

FIG. 9. Part of the secondary spongiosa of the tibia of a 3-day-old rat reacted for lactic dehydrogenase. Note the intense reaction in the osteoclasts. (× 350.)

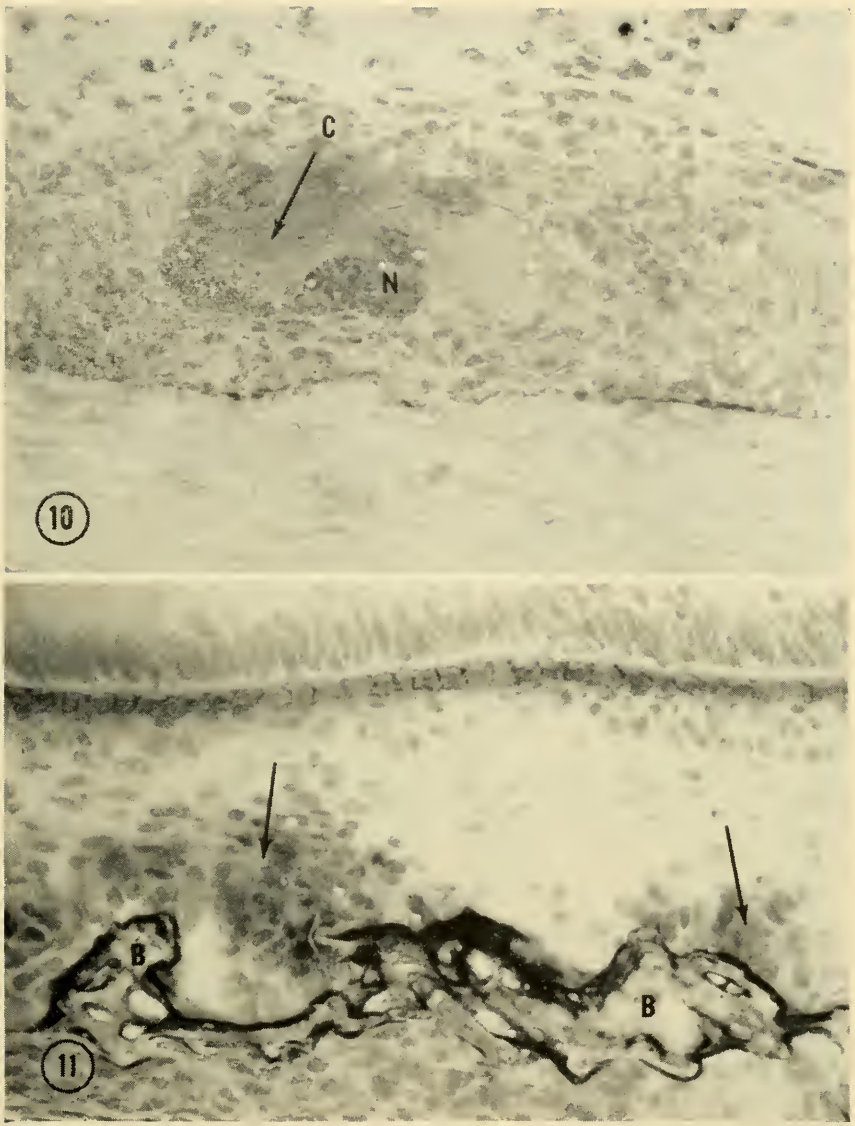

FIG. 10. Giant cell on implanted bone reacted for acid phosphatase and counterstained with hematoxylin. Activity was confined to the cytoplasm of the cell (*C*), while the mass of nuclei (*N*) stained with hematoxylin. (× 350.)

FIG. 11. Labial alveolar bone of a 3-day-old rat, taken from about the upper incisor tooth, reacted for acid phosphatase and counterstained with hematoxylin. Note activity in the osteoclasts (arrows) resorbing the bone (*B*). (× 350.)

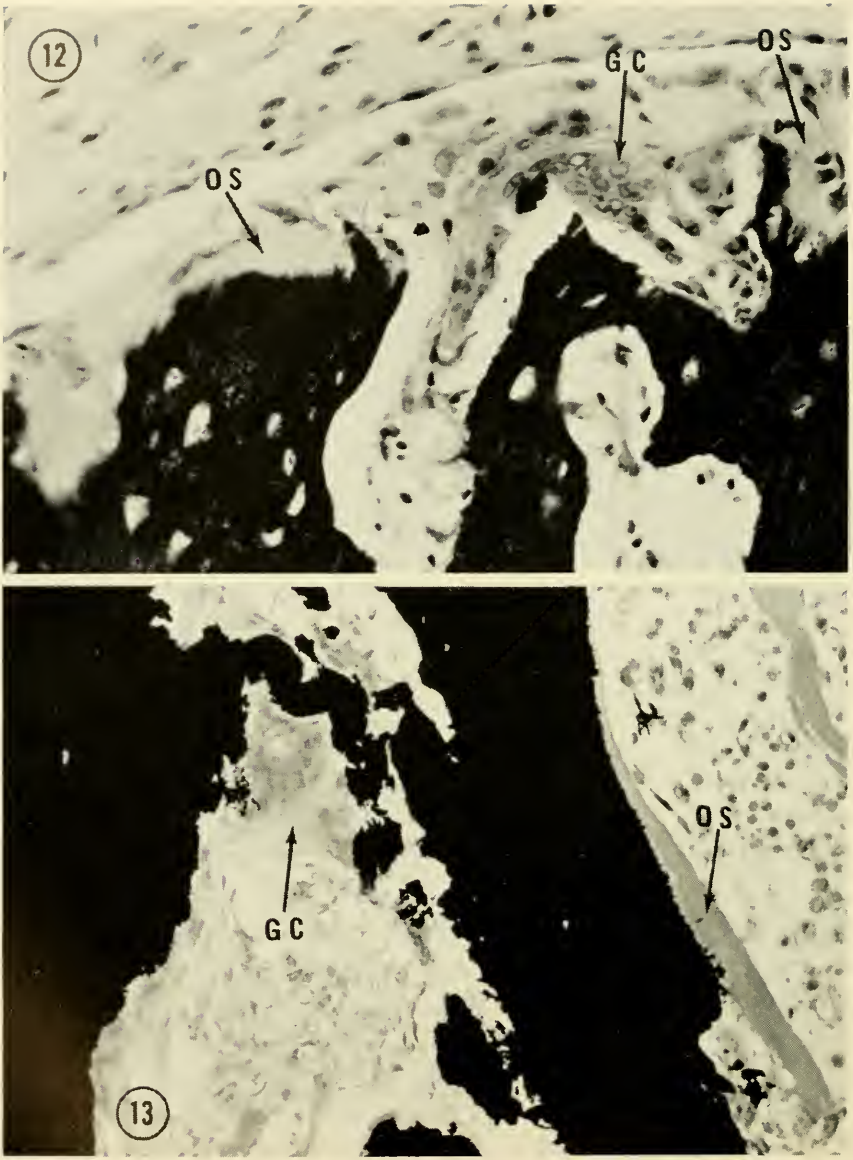

FIG. 12. Rachitic bone removed 1 week after implantation. Note giant cell (*GC*) on calcified bone but not on osteoid (*OS*). Gomori, hematoxylin and eosin. (× 350.)

FIG. 13. Rachitic bone removed 3 weeks after implantation. A giant cell (*GC*) is in contact with calcified bone, but not with osteoid (*OS*). Von Kossa, hematoxylin and eosin. (× 350.)

considerable activity. They commented that this high activity might be characteristic of multinucleated cells in general, since osteoclasts also had these enzymes. Incidentally, this high activity disposes of the views expressed by some writers that multinucleated cells are a degenerate and dying form. More recently, Cabrini *et al.* (1962) compared the histoenzymologic activity of osteoclasts with that of giant cells of foreign body granulomata and found intense activity of the following: acid phosphatase, succinic dehydrogenase, phosphoamidase, and β-glucuronidase in both types of cells. They commented that there was a high degree of metabolic activity in these cells and also that a similar enzymic pattern was needed for the absorption of different substances.

In the present experiments, cells were investigated that were actually attacking bone, either calcified or decalcified. The technique we used had the advantage that histological procedure could be kept to a minimum, and in particular histological decalcification was not required. The results showed that, when compared with "official" osteoclasts, our giant cells showed a close enzyme correspondence, differing qualitatively only in a somewhat lower acid phosphatase content. Though the evidence is circumstantial, presumably the mechanics of implant destruction and the enzymes involved are similar to those of osteoclasts found around bone. It is on this basis that we believe that our implants can be used as a model for the study of bone resorption, and that the giant cells are osteoclasts.

These osteoclasts obviously arose from the cells surrounding the implant and were observed in areas of cellular reaction. It is too early to say, in these investigations, what their precursors might be. Multinucleated cells seemed to spring into existence with remarkable rapidity, and cells with 2 or 3 nuclei were not commonly seen. Work using markers such as tritiated thymidine is planned in an endeavor to pin down the parents of the cells we are studying. A recent study (Fischman and Hay, 1962) offers convincing evidence that such cells, at least in the newt, may arise from blood cells. It would seem that the osteoclasts, which lack the ability to ingest trypan blue, are not part of the reticulo-endothelial system. It is possible that the intense initial mononuclear cell reaction may be related to the sub-

sequent appearance of osteoclasts, which arise from mononuclear cells by fusion.

The mode of action of osteoclasts on bone, that is, whether dissolution of collagen or of the inorganic phase is the first step, has been often debated. In a recent paper Takuma (1962) produced evidence that seemed to indicate that the inorganic phase was first removed, since matrix fibrils, denuded of crystals, could be seen at the bone edge in his electron micrographs. On the other hand, both Scott and Pease (1956) and Gonzales and Karnovsky (1961) interpreted their findings as showing that dissolution of collagen fibrils preceded phagocytosis of the crystals by the osteoclast. The present results do not clarify the question of the time sequence of osteoclast action, but they do show that the osteoclasts we have been studying are capable of attacking the organic matrix in the absence of the inorganic phase. There is no reason to doubt that this applies to osteoclasts in general.

It is the intention of the authors to expand this work into an investigation of the type of bone organic matrix that can undergo osteoclasis. The present results show that rachitic osteoid cannot be resorbed, a finding confirming that of Weinmann and Schour (1945) and of Bailie and Irving (1955). Cabrini *et al.* (1957) likewise found that predentin is immune to resorption during the internal resorption of dentin. Furthermore, since bone matrix can attract osteoclasts and be resorbed, even after decalcification, it would appear that the inorganic phase plays at most a minor role in causing osteoclasis. Our concept is that matrix that has once been calcified is resorbable and attracts cells to carry out this process. At present it is not known what this mechanism is, but possibly a chemical substance is liberated which provokes the process. There appeared to be a tropism to the ends of the implants where the bone had been cut. Sabin (1941) showed that phthioic acid extracted from the bodies of tubercle bacilli would evoke giant cell formation on injection. On the other hand, there may in addition be a chemical difference in the matrix after calcification which makes it resorbable. It is intended to alter the collagen of the implants by various chemical methods to see what effect this will have upon osteoclasis.

In our preliminary experiments, altering the parathyroid status

of the animals failed to influence the resorption of the implants. A possible explanation is that vital osteoblasts and osteocytes are necessary for this hormone to stimulate bone resorption.

Summary

1. Devitalized autogenous subcutaneous bone implants in rats have been studied with respect to osteoclasis.

2. Decalcified implants from normal rats began, after 2 weeks, to be resorbed by multinucleated giant cells. These cells had virtually the same enzyme activity as osteoclasts and were capable of resorbing uncalcified bone matrix.

3. The osteoid of devitalized autogenous implants from rachitic rats was not resorbed by giant cells until it had become calcified after implantation.

4. Parathyroid extract or parathyroidectomy did not affect the time of appearance or number of giant cells appearing around implants.

5. It is considered that the giant cells studied are comparable to osteoclasts. There appears to be a type of tropism influencing their appearance which is exerted by bone matrix that is or has been calcified, but not by matrix such as rachitic osteoid which has not been calcified.

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Resorption without Osteoclasts (Osteolysis)

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REMOVAL of minerals from the deeper regions of bone tissue has been postulated on the basis of morphological (Zawisch-Ossenitz, 1927; Heller-Steinberg, 1951; Ruth, 1954, 1961) and physiological observations (Talmage and Elliott, 1958; Talmage, 1962). Local changes in the organic matrix such as basophilia (Zawisch-Ossenitz, 1927; Ruth, 1954, 1961) or "depolymerization of the mucopolysaccharides" (Heller-Steinberg, 1951) have been associated with this phenomenon. More recently, bands of low density have been described in alpharadiographs (Bélanger and Bélanger, 1959) of demineralized trabeculae of rachitic (Vincent *et al.*, 1962) and norethandrolone-treated chicks (Bélanger and Migicovsky, 1961). A correlation between mid-trabecular low density of the matrix and basophilic staining was also established (Bélanger and Migicovsky, 1961).

The present various series of experiments cast some light on the nature of basophilia, on the mineral-organic interdependence, and also on the parathyroid–vitamin D–bone relationship.

Materials and Techniques

Animals

Chicks. Twenty-four chicks were raised on low-calcium–no vitamin D rachitogenic diet (Association of Official Agricultural Chemists, 1940) from the time of hatching and distributed as follows:

1. Six birds were given, after 1 week, a single oral dose of 1000 units vitamin D3.
2. Six birds received the same treatment and also 3 daily doses of 1 ml Para-Thor-Mone (parathyroid extract, Lilly, 100 units) intraperitoneally during the 2nd week.
3. Six birds were administered Para-Thor-Mone as above, without vitamin D.
4. Six birds were maintained as rachitic controls.

All birds were sacrificed on day 14, 24 hours after the administration of tracer. The tibiae were roughly dissected and fixed in 10 per cent neutral formaldehyde for 48 hours.

Rats. Twenty-four female Sprague-Dawley rats, of 150 to 200 gm body weight, were distributed and treated as follows:

1. Six rats after approximately 10 days of pregnancy were given 3 daily doses of 1 ml Para-Thor-Mone and killed on the 4th day.
2. Six nonpregnant rats were given 3 daily doses of 1 ml Para-Thor-Mone and killed on the 4th day.
3. Six rats of the same duration of pregnancy as those of group 1 but without Para-Thor-Mone were killed at the same time.
4. Six nonpregnant rats without Para-Thor-Mone were also killed as controls of group 2.

Dogs—Para-Thor-Mone. Fourteen young dogs of both sexes were treated as follows shortly after weaning:

1. One animal was injected with 100 units of Para-Thor-Mone intramuscularly for 3 successive days and killed on the 4th day.

One littermate without treatment was killed at the same time.

2. Five dogs were injected twice a day with 100 units of Para-Thor-Mone (total 600 units) and killed on the 4th day.

Five littermates without treatment were killed at the same time.

3. One dog was injected with 1000 units of Para-Thor-Mone and killed after 24 hours.

One dog was injected with 1000 units of Para-Thor-Mone and killed after 3 hours.

Dogs—EDTA. Ten normal dogs maintained on the regular stock diet were used. They were fasted overnight and anesthetized with nembutal. Blood samples of 5 ml were taken at 30-minute intervals, and the plasma was analyzed for calcium by a modification of the method of Fales (1953). After a 1-hour control period, bone biopsies were taken from skull, iliac crest, and tibial shaft. The dogs were then infused intravenously with an isotonic solution of disodium ethylenediamine tetraacetate (EDTA) in saline which had been adjusted to pH 7.4. The infusion was given at the rate of 40 ml per hour, and the concentration of EDTA was adjusted so that 5 to 15 mg calcium were chelated and removed per hour. Since the Ca-EDTA chelate is formed instantly and is physiologically inert, the calcium is removed from the physiological milieu immediately and as effectively as if it had been actually taken out of the body (Copp *et al.*, 1961). The effects produced are therefore due to the resulting hypocalcemia rather than to the EDTA. A typical curve showing the changes in plasma calcium and phosphorus during the course of the experiment is shown in Fig. A. It should be noted that the plasma calcium was determined by a method which does not include the EDTA-bound calcium. The curve for this animal indicates that under the stimulus of hypocalcemia and increased endogenous parathormone production the dog was able to more than balance the removal of 7.5 mg Ca/kg/hour or 2.2 gm over a 14-hour period. After 12 hours of EDTA infusion, bone biopsies were taken from the opposite side of the body to correspond to the original control samples. It was therefore possible to compare bone from the same animal before and after the period of active calcium mobilization.

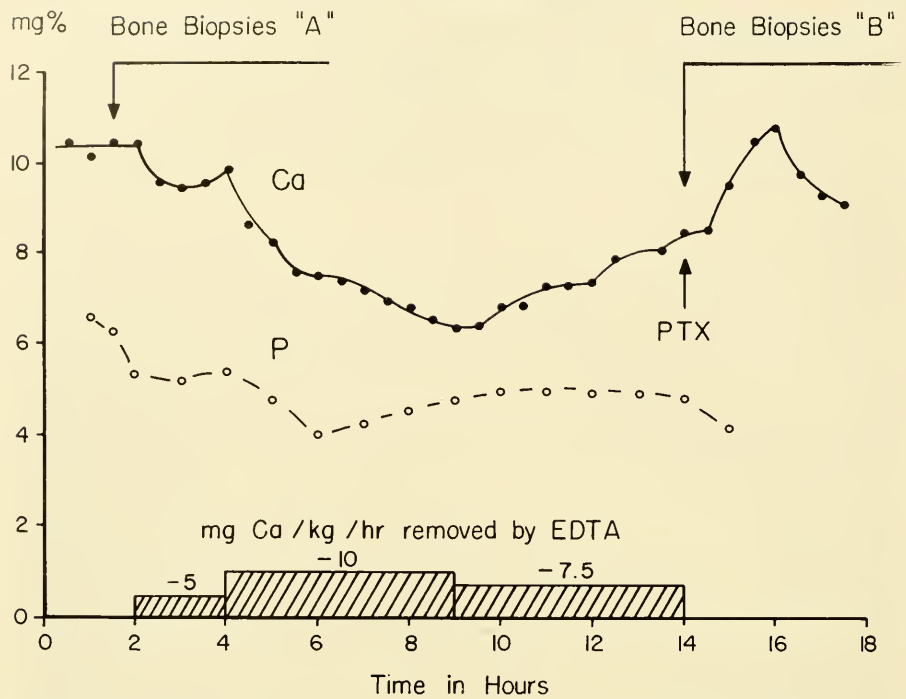

FIG. A. Changes in plasma calcium and phosphorus during intravenous infusion of EDTA into a 23-kg dog. The infusion removed 2.2 gm calcium from blood during the 12-hour period, inducing hypocalcemia and presumably stimulating endogenous parathormone production. The initial control bone biopsies were taken as indicated at "A" and the postinfusion bone biopsies were taken at "B." Parathyroidectomy was performed as indicated by PTX.

Histology

CHICKS AND RATS. One bone from each of the chicks and rats of all groups was demineralized in EDTA as above. The other tibia was embedded in low-viscosity nitrocellulose without demineralization.

DOGS. Portions of the metaphysis of the tibia and a portion of the parietal bone as well as a variety of other bones in group 3 were cut with a band saw and fixed for 24 to 48 hours in neutral formaldehyde.

Pieces of these specimens were then embedded in acrylic resin for microradiography (Bélanger *et al.*, 1963). Comparable pieces were demineralized in EDTA or in nitric ethanol (nitric acid 5 per cent in 70 per cent ethanol) and then embedded in paraffin.

Alpharadiographs (Bélanger and Bélanger, 1959) of 10-micron nonradioactive demineralized sections were obtained on Eastman Kodak spectroscopic plates, type V-O and type 649-O. Alpharadiographs of 12-micron mineralized sections were recorded on Di-Noc Contrast II plates (Di-Noc Chemical Arts Ltd., Honeoye, New York).

Adjacent mineralized sections were also submitted to x-ray microradiography with a Philips 5-kv apparatus and the results were recorded on Eastman Kodak film type 649-O.

Series of mineralized and demineralized sections of the tibiae were prepared for phase contrast microscopy in Permount (Fisher), without stain.

Series of demineralized sections were stained by the Masson trichrome stain with light green (Masson, 1929), with toluidine blue (Bélanger and Hartnett, 1960), by the Hale iron adsorption method for acid mucopolysaccharides (Hale, 1946), by the Hotchkiss-McManus periodic acid–Schiff method (Gomori, 1952) for neutral polysaccharides (Hooghwinkel and Smits, 1957; Leblond *et al.*, 1957), and by the combined Alcian blue–PAS stain of Mowry (1956).

Observations

Chicks

Staining. Bone sections stained by the *Masson trichrome* show areas which take up the red dyes and other sites which are green acceptors. The material of the present series has revealed definite patterns of staining apparently related to treatment. The trabecular matrix and also the characteristically thick periosteum of rachitic birds were stained almost exclusively green (Fig. 1). This statement applies at least to matrix formed during the nutritional experiment.

In birds treated with Para-Thor-Mone (Fig. 2), the trabeculae were thicker and mostly green. However, the more mature trabeculae

located on the marrow side of the diaphysis showed progressively extensive red patches (Fig. 2).

The chicks which received vitamin D showed a predominance of red staining, beginning early, at the site of growth (Fig. 3), i.e. close to the periosteum. Those which received both vitamin D and Para-Thor-Mone produced thin trabeculae which were stained green close to the periosteum, then red. The more centrally located ones were green at the immediate border (immature bone or osteoid), then red, then green again in the mid-trabecular region (Fig. 4).

Acid mucopolysaccharides. The osteocytes particularly in all chicks which received vitamin D were different in size and also in staining properties (Figs. 11 and 12). Close to the periphery of the trabecula, the cells and lacunae were small and poorly stained. They seemed to enlarge progressively toward a maximum size in the center of the trabeculae, where the cytoplasm of the osteocytes exhibited toluidine blue metachromasia, azurophilia (Fig. 11), and a positive Hale reaction (Fig. 12). The matrix immediately surrounding the lacuna was also metachromatically stained (Fig. 11). The adjacent matrix did not show metachromasia, but basophilia (Fig. 11) progressively decreasing away from clusters of large osteocytes (Fig. 11). The Hale reaction was localized to the cytoplasm and to the processes of the large osteocytes (Fig. 12).

Tissues which were demineralized in nitric-ethanol apparently retained larger amounts of the metachromatic, Hale-positive, and alcianophilic material than tissues demineralized in EDTA. In both cases, however, the localizations were similar.

In chicks treated with Para-Thor-Mone and vitamin D, several of the enlarged lacunae located in the mid-trabecular region either were

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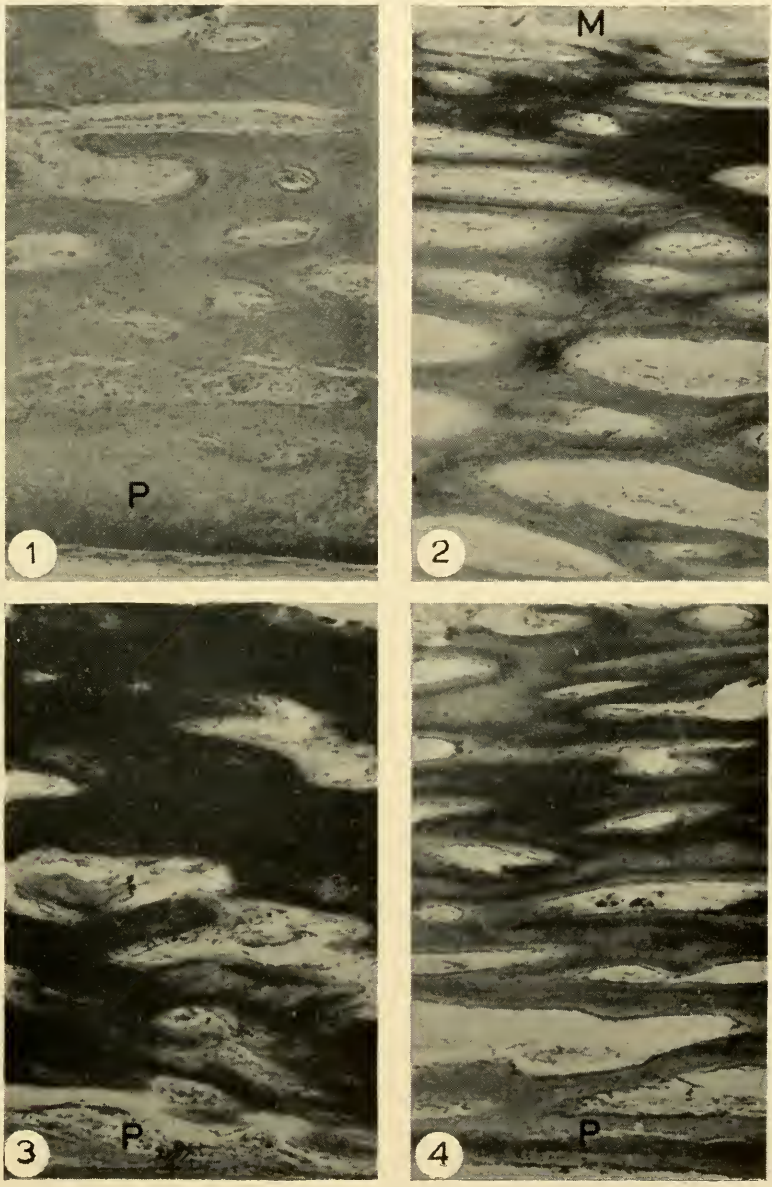
FIGS. 1 TO 4. Masson-stained EDTA-demineralized sections of mid diaphysis of 3-week-old chicks. (× 88.)

Fig. 1. On rachitogenic diet.
Fig. 2. On rachitogenic diet and Para-Thor-Mone treatment.
Fig. 3. On rachitogenic diet and vitamin D treatment.
Fig. 4. On rachitogenic diet and vitamin D–Para-Thor-Mone treatment.

The periosteum (*P*) is in the lower part of the pictures. The marrow side (*M*) is uppermost.



FIGURES 1 TO 4

empty or showed osteocytes which were apparently degenerate (Fig. 12).

Periodic acid–Schiff. Sections of EDTA-demineralized bone revealed a more intense and less uniform distribution of PAS-stained material than sections demineralized in nitric-ethanol.

The bones of rachitic and Para-Thor-Mone–treated birds were the least stained. The trabeculae revealed an unstained or poorly stained band at the periphery (osteoid, prebone) and a more intensely stained axial region, containing the large osteocytes. The osteoblasts and osteocytes were generally weakly stained if at all.

The vitamin D–treated chicks and those which received Para-Thor-Mone along with vitamin D showed a generally more intense PAS stain. The trabeculae contained three zones which were unevenly distributed: a peripheral weakly stained border, then a more or less wide band of higher intensity, and eventually a mid-trabecular island of more weakly stained tissue around the hypertrophic osteocytes. These zones were less apparent in birds which received only vitamin D. In some areas, the central clear islands contained masses of PAS-stained material of irregular shape and size.

Sections stained by the *Alcian blue–PAS* method showed concentrations of alcianophilic material in the large osteocytes and adjacent matrix of the mid-trabecular region, particularly evident in the Para-Thor-Mone–vitamin D group and in sections which had been demineralized in EDTA.

Unstained sections under phase contrast revealed marked differences between the various groups at the accretion sites of bone and cartilage. These will be reported elsewhere. However, no characteristic variations in pattern could be detected within the older regions.

Alpharadiographs. Alpharadiographs of demineralized sections produced patterns of varied densities. In trabeculae of rachitic birds (Fig. 5), the matrix appeared almost uniformly dense with apparently empty holes marking the lacunae, and among them only a few of larger size could be seen (Fig. 5).

In birds treated with Para-Thor-Mone (Fig. 6) the larger lacunae

were more numerous, and in their immediate vicinity the organic matrix appeared less dense.

Chicks which received vitamin D also showed mid-trabecular islands of large lacunae and low-density matrix (Fig. 7).

The most extensive zones of mid-trabecular low density were registered in birds treated with vitamin D and Para-Thor-Mone combined (Fig. 8).

Alpharadiographs of mineralized sections (Figs. 9 and 10) recorded on a high-contrast emulsion revealed a loss of minerals parallel to the loss of organic density in mid-trabecular portions of the vitamin D–treated birds, and a considerably more extensive low density in the vitamin D–Para-Thor-Mone group.

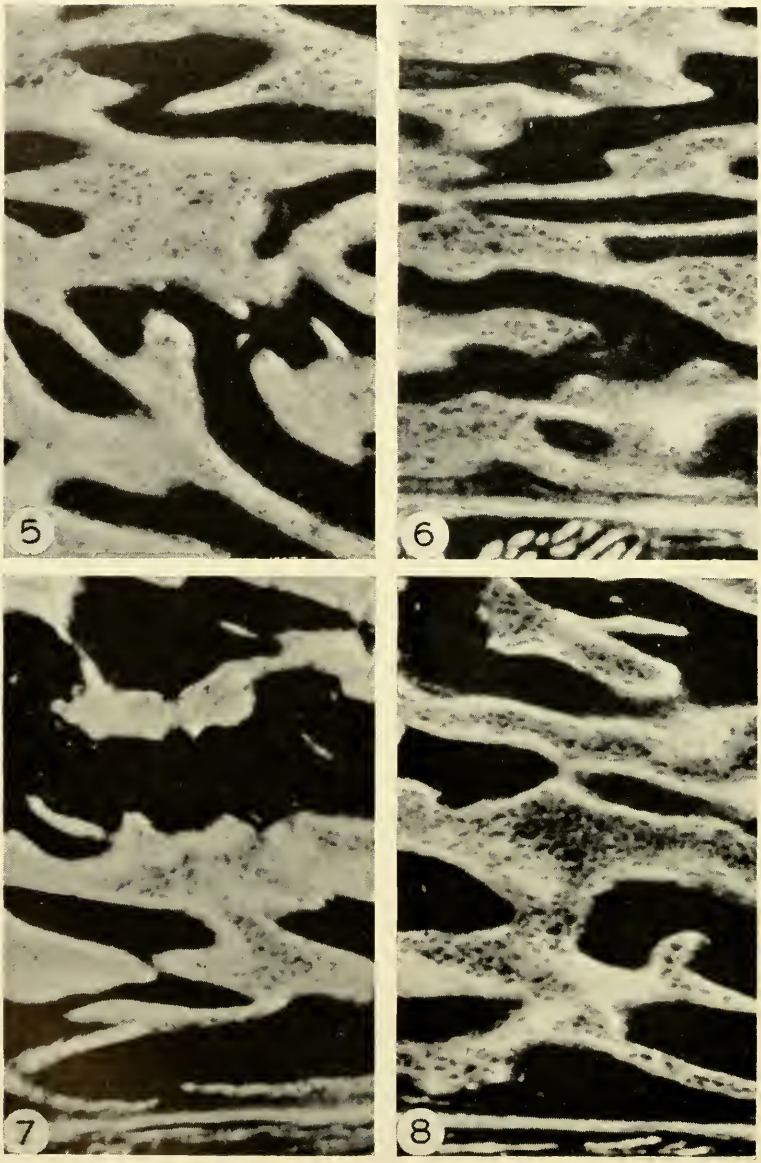
X-ray microradiography produced comparable results.

Norethandrolone. Alpharadiographs of the demineralized tibia of norethandrolone-treated chicks have previously been described (Bélanger and Migicovsky, 1961). Staining with toluidine blue and also with a combination of neutral red and fast green (Fig. 16) revealed that some of the hypertrophic osteocytes actually acquired all the staining behavior of cartilage cells and were also apparently responsible for a large concentration of acid mucopolysaccharides in the surrounding matrix.

Rats

TOLUIDINE BLUE. EDTA-demineralized bones revealed considerable variations among the four groups. The diaphysis of the control animals (Fig. 13) contained mostly small osteocytes. However, a band of larger cells was located about midway between the periosteum (Fig. 13, *P*) and the border of the central marrow cavity. The hypertrophic osteocytes in that area had a metachromatic-staining cytoplasm when stained with toluidine blue. The immediate border of their lacunae (capsule) exhibited the same tinctorial property.

Rats injected with Para-Thor-Mone (Fig. 14) showed an increase in the number of hypertrophic and metachromatic osteocytes. These extended from the center of the diaphysis inward and consequently were the older cells. The organic matrix was lightly basophilic around most of these hypertrophic osteocytes (Fig. 14). Elsewhere in the



FIGURES 5 TO 8

sections, the lamellar arrangement of the bone was made apparent by the presence of alternating bands of more and less stained matrix (Fig. 14).

Rats which were 10 to 12 days pregnant revealed a similar picture but with great variations from one animal to another.

Pregnant rats injected with Para-Thor-Mone (Fig. 15) revealed an exaggeration of the above-described phenomenon, i.e. a greater proportion of hypertrophic osteocytes, surrounded by strongly basophilic matrix.

ALPHARADIOGRAPHY. Islands of low density were observed midway between the periosteal and marrow borders of the diaphysis or closer to the marrow border.

It was evident that the areas of low density in the alpharadiographs matched the basophilic matrix and concentrations of hypertrophic osteocytes in the stained sections (Figs. 14 and 15).

Dogs

PARA-THOR-MONE. *X-ray microradiography.* The trabecular bone of normal dogs revealed the presence of a denser white band in the near center (Fig. 17). Some lacunae in this area appeared wider than at the periphery. Some of these were also closer to one another; some were even confluent (Fig. 17). In compact bone, larger lacunae were observed in the interstitial lamellae and also at the periphery of some of the bigger osteons.

In the dog which received 100 units of Para-Thor-Mone, it was not possible to detect any significant change in the microradiographic pattern.

In those which received 600 units over 3 days, there was no important modification of the image in sections of the tibia. However,

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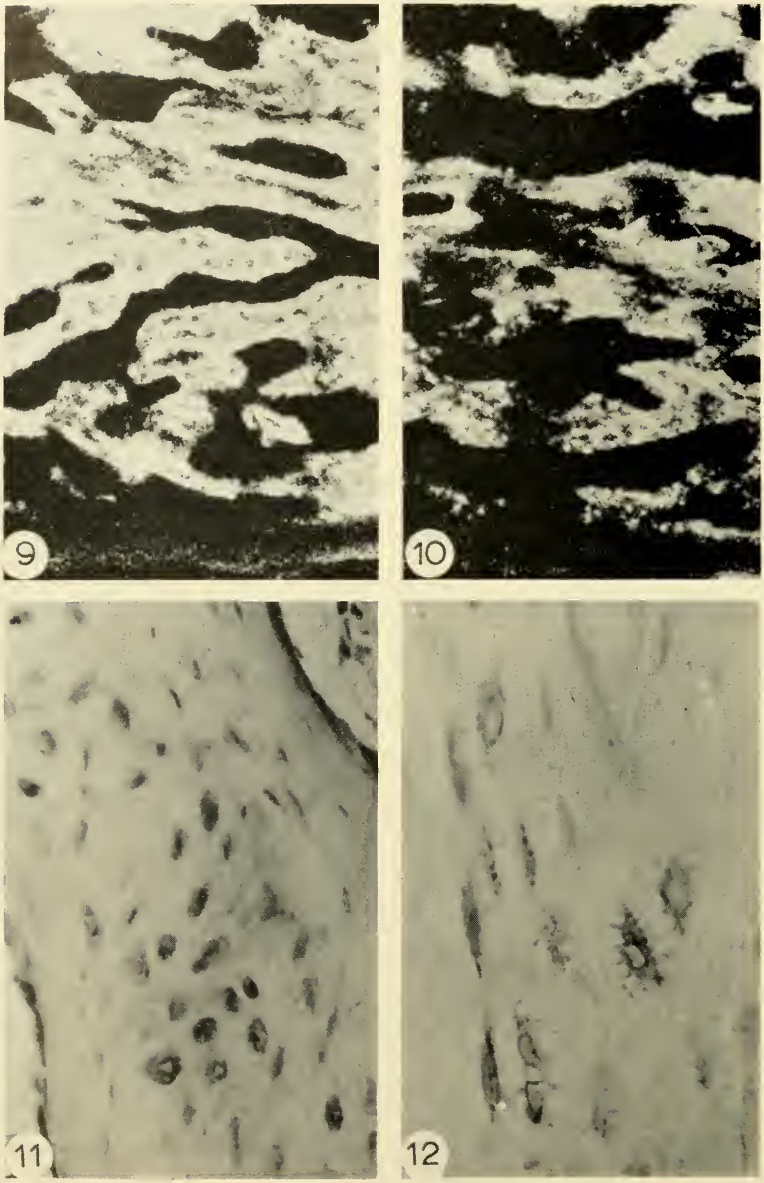
FIGS. 5 TO 8. Alpharadiographs from sections as above. (× 144.)

Fig. 5. Rickets.
Fig. 6. Rickets plus Para-Thor-Mone.
Fig. 7. Rickets plus vitamin D.
Fig. 8. Rickets plus vitamin D–Para-Thor-Mone.

The periosteum is in the lower part of the pictures.



FIGURES 9 TO 12

the skull revealed islands of large mid-trabecular lacunae accompanied by decreased matrix density (Fig. 18).

The dog which was treated with 1000 units of hormone 24 hours before sacrifice showed a considerable extension of lacunar enlargement and confluence (Fig. 19) as well as adjacent loss of density. These changes were present in both the skull and the tibia samples but they were more extensive in the former.

Alpharadiography of demineralized sections revealed some surprising facts. (1) In the normal tibia, the periosteal lining was denser than the adjacent compact bone matrix. (2) In the osteons, the central portion was generally denser than the periphery (Fig. 20). (3) The width of dense tissue varied from one osteon to another (Fig. 20). (4) Alternating bands of high and low density were observed in the osteons and in the interstitial lamellae (Fig. 22). (5) Irregular zones of low density accompanying enlarged lacunae were present mostly in interstitial tissue.

In the trabecular bone of the skull, these zones occupied a mid-trabecular position as previously described in the chick (Bélanger and Migicovsky, 1961; Vincent *et al.*, 1962).

The bones of Para-Thor-Mone–treated dogs showed a coincidence between the low-density mid-trabecular and interstitial areas of the microradiographs (Fig. 18) and alpharadiographs (Fig. 20). It seems, however, that the areas of lower density were more extensive in the demineralized sections.

Demineralized *stained sections* of the above-mentioned material showed changes which were comparable to those previously reported in the chick. Prominent in the skull and tibia specimens of the dog killed 24 hours after 1000 units of Para-Thor-Mone, these were: (1) an enlargement of the osteocytes in the sites of resorption,

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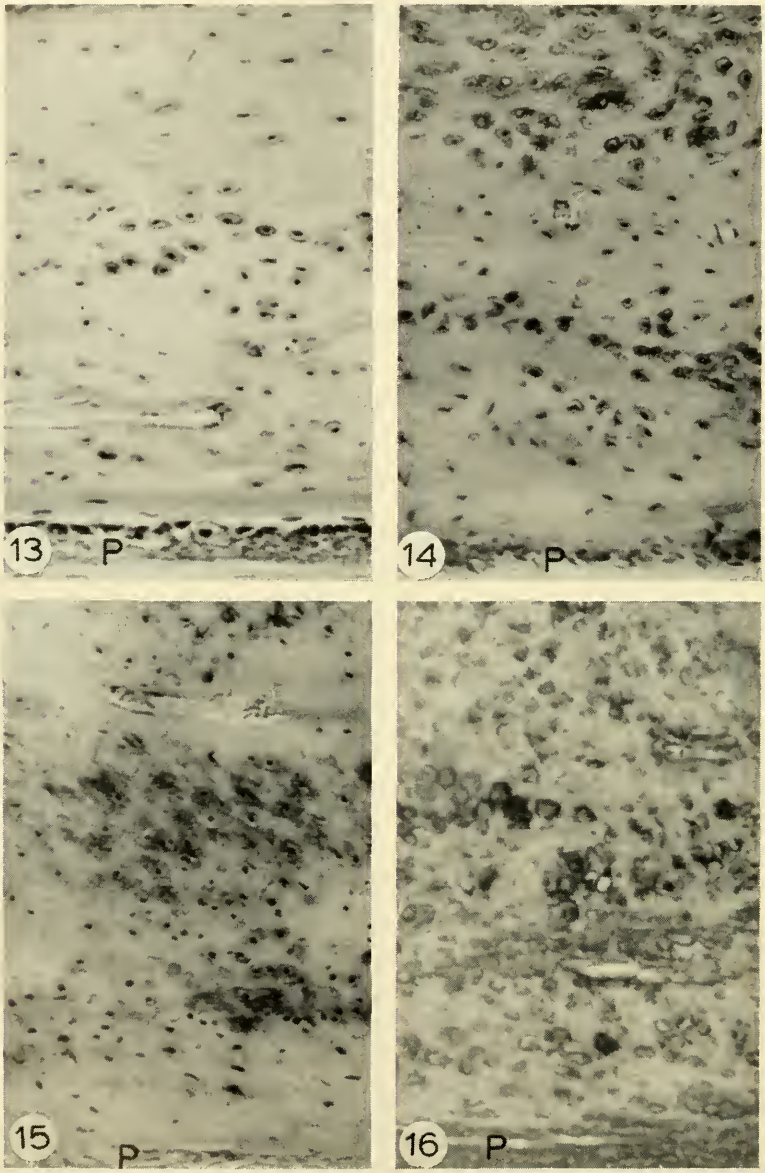
FIGS. 9 AND 10. Two aspects of periosteal bone in alpharadiographs of mineralized celloidin sections from chicks treated with Para-Thor-Mone and vitamin D. The periosteal border is in the lower part of the picture. (× 88.)

FIG. 11. Large mid-trabecular osteocytes surrounded by basophilic matrix in section from vitamin D–treated chick. Diluted Wright stain. (× 280.)

FIG. 12. Similar location to that of Fig. 11, in section from vitamin D–Para-Thor-Mone–treated bird. Hale stain. The cytoplasm and processes of the enlarged osteocytes are stained. Notice exhausted cells. (× 748.)



FIGURES 13 TO 16

accompanied by the presence of intracellular and extracellular metachromatic material after toluidine blue or azur I staining; (2) large patches of centro-trabecular or interstitial basophilic or metachromatic material corresponding to the sites of resorption revealed by x-ray microradiography and alpharadiography.

EDTA VENOCLYSIS. In normal dogs, it is possible to remove a considerable amount of calcium from blood by continuous intravenous infusion of EDTA, and the blood calcium is rapidly restored to the normal level after the infusion has stopped (Copp, 1957). Copp and Davidson (1961) have shown that perfusion of the parathyroid glands with hypocalcemic blood (8 mg % Ca) appears to release increased amounts of endogenous parathormone, which results in a rise in systemic blood calcium presumably due to increased mobilization of this element from bone. This mechanism would account for the prompt replacement of calcium removed by continuous intravenous infusion of EDTA.

As previously reported, thin sections of undemineralized dog bones, cancellous as well as compact, revealed regional sites of higher density. These were associated with the presence of enlarged and sometimes confluent lacunae (Fig. 21). Islands of lower than average density, also containing enlarged lacunae (Fig. 21), were observed in x-ray microradiographs. Corresponding islands of low

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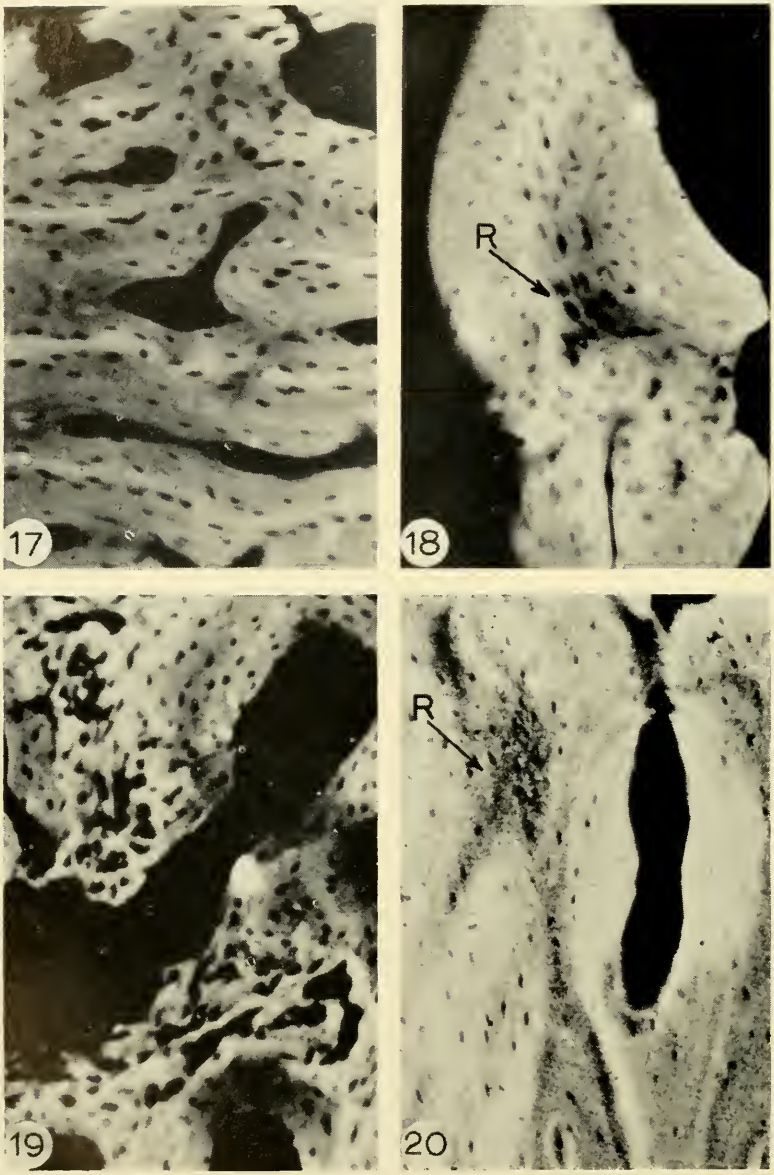
FIG. 13. Part of diaphysis of the tibia from a control rat. A mid-diaphyseal band of large osteocytes shows intracellular and extracellular (capsule) metachromatic material. *P*, periosteum. Toluidine blue stain. (× 144.)

FIG. 14. Same, from a rat injected with 300 units of Para-Thor-Mone over 3 days. The number of large osteocytes is greatly increased. Basophilic ground substance is present around these. The matrix is lamellar. *P*, periosteum. Toluidine blue. (× 144.)

FIG. 15. Same, from a pregnant rat injected with Para-Thor-Mone as above. Large numbers of hypertrophic and metachromatic osteocytes are surrounded by intensely basophilic matrix. *P*, periosteum. Toluidine blue. (× 144.)

FIG. 16. Part of the diaphysis from a 3-week-old chick fed norethandrolone (Nilevar, 0.5 gm/kg) for a week. A large number of hypertrophic osteocytes are present; some are surrounded by strongly neutral red–positive matrix and look altogether like chondrocytes. *P*, periosteum. Neutral red–fast green. (× 144.)



FIGURES 17 TO 20

density were observed in alpharadiographs of demineralized tissues (Fig. 22, arrows).

In alpharadiographs, parallel bands of high- and low-density lamellae could also be seen (Fig. 22). These have been previously observed by Rouiller *et al.* (1952) and Frank *et al.* (1955) with the electron microscope, and also by Vincent and Mandiangu (1958) by x-ray microradiography. The border of the trabeculae as well as the periphery of the osteonic canals were sharply defined by the presence of high-density material (Fig. 22).

Following intravenous administration of EDTA, the older compact bone (tibia) was little affected. However, more recently formed compact bone (Figs. 23, 24, and 26) and also cancellous bone to a greater extent (Fig. 25) revealed an increase in the size and frequency of lower-density areas. These were associated with the presence of enlarged, confluent lacunae (Figs. 23 and 25). In the immediate vicinity of these, sites of lower density were occasionally recognized (Fig. 25) as distinct from the lacuna itself. Comparably located islands of low density were recorded by alpharadiography of the demineralized sections (Figs. 24 and 26).

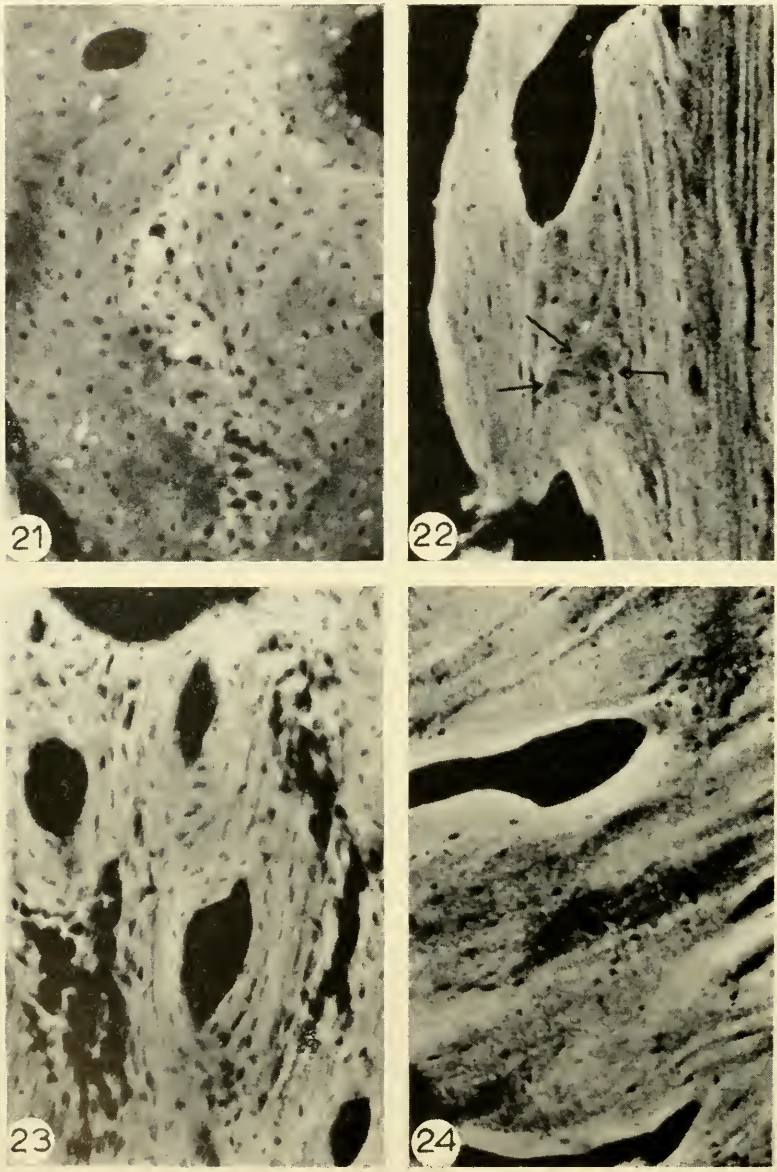
Demineralized sections of the skull, stained with toluidine blue, showed a concentration of metachromatic material in and around the hypertrophic osteocytes in the islands of low density, as previously reported in Para-Thor-Mone–treated dogs.

Fig. 17. Trabecular bone in tibia of young dog. Notice some larger confluent lacunae in dense mid-trabecular region. X-ray microradiograph. (× 144.)

Fig. 18. A skull trabecula from a dog treated with 100 units Para-Thor-Mone twice a day for 3 days. Notice more numerous large lacunae and surrounding low density of matrix in area of resorption (*R*). X-ray microradiograph. (× 144.)

Fig. 19. Part of the skull of a dog killed 24 hours after a single dose of 1000 units Para-Thor-Mone. Many enlarged, confluent lacunae are present. X-ray microradiograph. (× 144.)

Fig. 20. A portion of compact bone from the tibia of a dog treated with 100 units Para-Thor-Mone twice a day for 3 days. Notice high density of organic matrix in immediate border of large and small haversian canals; also enlarged lacunae and low organic density in resorption area (*R*). Alpharadiograph of a demineralized section. (× 88.)



FIGURES 21 TO 24

Humans

A variety of pathological material was obtained and submitted to x-ray microradiography, alpharadiography, and histochemical staining. The results were in accord with those of our animal series.

A specimen of bone with spontaneous fractures from a case of parathyroid adenoma revealed the presence of enlarged confluent lacunae accompanied by decreased density of matrix (Fig. 27).

A biopsy from a patient suffering from osteogenesis imperfecta also showed, in x-ray microradiographs of mineralized sections, enlarged confluent lacunae and adjacent low density of matrix (Fig. 28).

Specimens of neoplastic bones (osteogenic sarcoma, Ewing's sarcoma) and specimens of Paget bone revealed similar instances. In this latter case, however, the density records are complex and will be the subject of a further report.

Discussion

The Normal Phenomenon

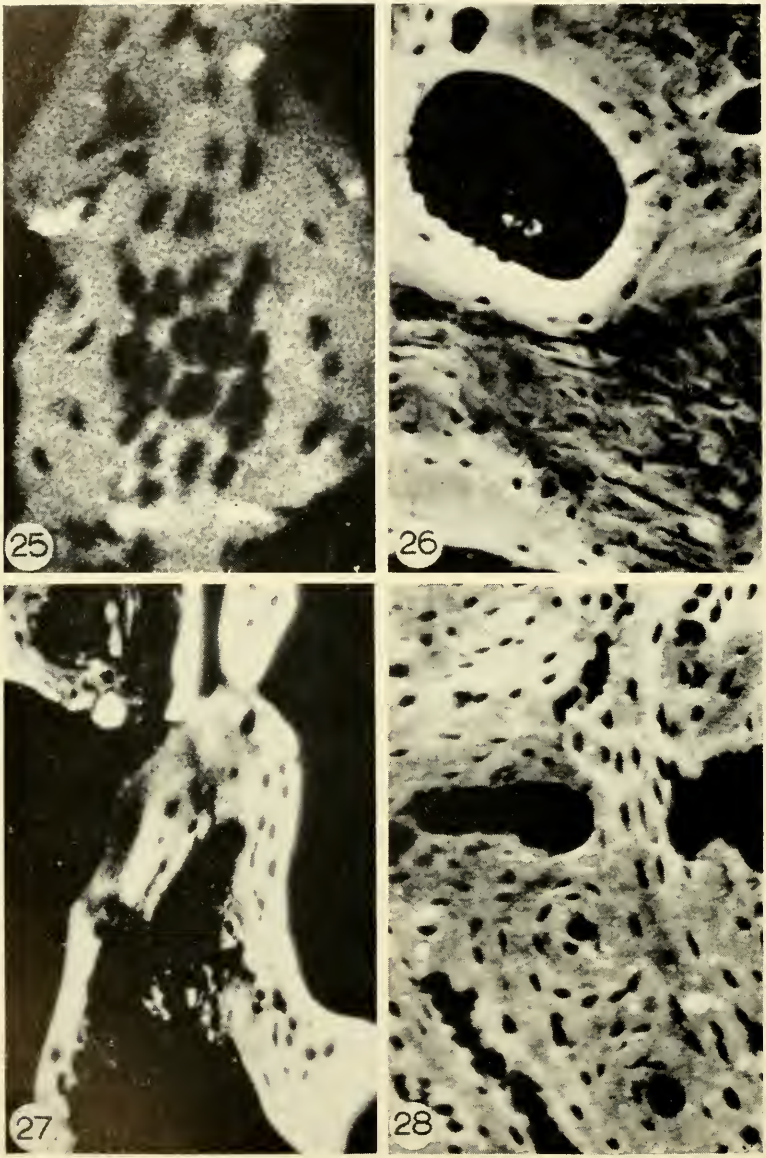
It appears from the present series of experiments that there are in chicks, rats, and dogs osteocytes of two different types. The *small osteocytes* (Figs. 11 and 13) are predominant near the formative surfaces. These cells have a cytoplasm which stains weakly with toluidine blue. The organic matrix surrounding the small osteocytes has a high density as demonstrated by alpharadiography (Fig. 22);

Fig. 21. A portion of compact bone (tibia) of a control dog showing interstitial lamellae of variable density and lacunae of different sizes. X-ray microradiograph. (× 144.)

Fig. 22. Compact bone of control dog, showing alternate bands of high and low density, lacunae of different sizes, and small low-density areas (arrows) around some of the larger lacunae. Alpharadiograph of demineralized section. (× 88.)

Fig. 23. A portion of compact bone (skull) of an EDTA-infused dog. Large, confluent resorption lacunae. X-ray microradiograph. (× 144.)

Fig. 24. Alpharadiograph of a comparable demineralized specimen. The immediate vicinity of the haversian canal shows high density and enlarged lacunae. Islands of loss of organic density correspond to those of low salt concentration in Fig. 3. (× 144.)



FIGURES 25 TO 28

its salt content as estimated by x-ray microradiography is not uniform (Fig. 21).

The *large osteocytes* (Figs. 11 to 13), more centrally located and older, show a hypertrophy which is mostly cytoplasmic. These cells contain material which induces the phenomenon of metachromasia with toluidine blue. The capsule which surrounds their lacuna contains similar material which can be stained by azur, Alcian blue, and neutral red. The adjacent organic matrix contains little or no metachromatic material under normal conditions.

X-ray microradiographs have revealed that there is generally a low concentration of bone salt around the hypertrophic osteocytes. Engfeldt and Zetterström (1954) and Engfeldt *et al.* (1956) interpreted this phenomenon as "newly formed areas associated with resorption cavities, in which the osteocytes are often surrounded by a large irregular zone containing small amounts of mineral salt or none at all."

Heller-Steinberg (1951), Rutishauser and Majno (1951), and also Lipp (1954) have recognized changes in the mucopolysaccharide content of the lacunae and their border which they have considered to be associated with resorption.

Alpharadiography of demineralized bone sections has revealed the surprising fact that the youngest part of the trabeculae or of the osteons (Figs. 8, 20, 22, and 24) is the densest organic part. It has also shown that in the more mature fraction, islands of low density containing large lacunae correspond to the areas of low mineral content which appeared in the x-ray records.

FIG. 25. Lacunar resorption in trabecular bone (skull). The enlarged lacunae are surrounded by occasionally confluent areas of low density. X-ray microradiograph. (× 180.)

FIG. 26. Alpharadiograph of a demineralized section of a portion of cranium from a dog subjected to EDTA venoclysis. Notice dense border of osteonic canal; lower density and fibrillar appearance of interstitial area underneath; enlarged lacunae and distended canaliculi, upper right. (× 110.)

FIG. 27. A portion of human diaphyseal humerus from a patient with parathyroid adenoma. Notice enlarged lacunae and adjacent low-density matrix. Alpharadiograph of demineralized section. (× 110.)

FIG. 28. A portion of diaphyseal bone from a child suffering from osteogenesis imperfecta. Notice numerous enlarged and confluent lacunae and low bone density. X-ray microradiograph of mineralized section. (× 110.)

These combined facts lead to the concept of a dual role for the osteocytes. While the young and small osteocyte probably continues at a slower pace the accretion initiated by the osteoblast, *the mature, hypertrophic osteocyte presides over salt removal through changes induced in the organic matrix* (osteolysis).

A Variety of Factors Which Induce Osteolysis

Pregnancy in the rat, as observed by Ruth (1954) and now confirmed, accelerates osteolysis. Para-Thor-Mone, vitamin D, and norethandrolone are all acceleration factors. We have also observed that toxic substances such as lathyric seed and fluoride promote this phenomenon. A few samples of human bone neoplasms recently obtained, and also specimens from two children afflicted with osteogenesis imperfecta, have revealed exaggerated osteolysis.

Under any one or a combination of these factors, the maturation of the osteocyte has been accelerated (Figs. 14 to 16) and the secretory mechanism has been stepped up. Metachromatic material has been deposited sometimes homogeneously, in a decreasing gradient around the lacunae, sometimes as irregular granular clumps.

Hyperactive large osteocytes in our experimental material have oftentimes become depleted (Fig. 12) and died. Death of the osteocytes has been reported by several investigators. It has been related to a variety of injuries: rickets, osteomalacia, chronic phosphorus and thallium intoxication, fluorosis (Rutishauser and Majno, 1951); osteolathyrism (Selye, 1957); beryllium intoxication (Kelly *et al.*, 1961); circulatory insufficiency (Sherman and Selakovitch, 1957); old age (Frost, 1960).

X-ray microradiography of mineralized sections and alpharadiography of comparable demineralized matrix have revealed that *concomitant* accelerated loss of mineral and organic density occurred in the immediate vicinity of the hypertrophic lacunae (Figs. 6 to 10, 18 to 20, and 23 to 26). Some of these records indicate that the osteolytic phenomenon is related to single cells or to small groups of adjacent enlarged osteocytes (Figs. 22, 27, and 28).

The fact that mineralization of the lacunae appears as "subsequent to cell death" (Kelly *et al.*, 1961) or as "characteristic of aging"

(Frost, 1960) indicates that osteolysis is the result of a vital function of the mature, hypertrophic osteocyte.

The Mechanism of Osteolysis

The facts reported here most likely reveal only a small part of the mechanism of osteolysis. It appears that this property of bone, not clearly defined as yet, occurs *through changes which take place primarily in the organic matrix.* These changes lead to a loss of density, partly explained by the secretion of acid mucopolysaccharides. The loss of salt follows the modification of the organic substrate.

Although it was not within the realm of the present enterprise to demonstrate this fact, it is highly probable that collagen as well as the mucopolysaccharides is involved in osteolysis, through a process of enzymic degradation generated by the hypertrophic osteocytes. Rutishauser and Majno (1951) have already reported that alkaline phosphatase activity which was present in osteoblasts, and which had temporarily disappeared in the small osteocytes, reappears in the large osteocytes. More recently, Lipp (1959) has observed the occurrence of leucine aminopeptidase in "osteoclasts, osteoblasts and osteocytes . . . only those which reveal resorption within their lacunae." Finally, the removal of hydroxyproline from bones after peritoneal lavage has just been reported by Talmage (1962). This author states that hydroxyproline removal follows a pattern quite similar to that of the mineral components previously studied.

Summary

In the older regions of cancellous and compact bone from chick, rat, dog, and man, large osteocytes which secrete acid mucopolysaccharides were observed.

Their cytoplasm, the capsule of their lacuna, and the adjacent matrix were positive to different histochemical stains for acid mucopolysaccharides.

These cells were located in areas which showed low density in x-ray microradiographs of mineralized sections and in alpharadio-

graphs of demineralized sections. These areas appeared consequently to be sites of resorption (osteolysis).

Osteolysis was enhanced by pregnancy, also by administration of Para-Thor-Mone, norethandrolone, and vitamin D. It was strongly stimulated by EDTA venoclysis. Exaggerated osteolysis was observed in osteolathyrism and fluorosis of rat; also in parathyroid adenoma, osteogenesis imperfecta, bone neoplasms, and Paget's disease of man.

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21

In Vitro Studies of Bone Resorptive Mechanisms

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IT IS clear that resorption of bone takes place under a variety of normal and pathological circumstances; yet knowledge of the details of the process, its control, and its relationship to other systems for the removal of unwanted tissue elements is still fragmentary. Though changes in cellular morphology on the one hand and in the morphology and physiology of the mineralized extracellular matrix on the other have been shown to occur in bone undergoing resorption, the relation between these two at the chemical level has remained obscure despite extensive investigation (McLean and Urist, 1961; Lacroix, 1956; Jowsey, 1960; Gaillard, 1961; Frost *et al.*, 1960; Vincent and Haumont, 1960).

Any comprehensive review of what has been done is beyond the scope of this paper. Therefore, I shall discuss largely our own work, indicating how our thinking about the chemistry of bone resorption has developed, outlining the ways in which we have attempted to study the process at the biochemical level, and reviewing our progress, such as it is, along these lines. The experiments to be described began four years ago and have been carried out with a number of collaborators including Drs. Nancy Nichols, André Borle, Stein Schartum, Gilbert Vaes, Barry Flanagan, and John Woods. Without

their imagination, skill, and hard work little would have been accomplished.

Either modification or disorganization-and-removal of the complex of heavily calcified collagen, ground substance, and cells which we call bone is required for the successful accomplishment of several phenomena of great import in the life of many species. One or both are required for the maintenance of calcium and phosphorus concentrations in the circulating fluids, for the remodeling of the skeletal structures during growth, for the internal remodeling of compact bone, and for the removal of bone irretrievably damaged by disease or trauma. The first of these phenomena, at least in theory, need involve only a mechanism for the mobilization and removal of some of the mineral phase of the tissue. On the other hand, the last three involve the total simultaneous removal of three separate types of extracellular material—mineral salts, collagen, and the polysaccharides of the ground substance—as well as the cells themselves. Moreover, it seems likely that, with the possible exception of the removal of dead bone, the mechanisms for bone resorption are endogenous to the bone itself and are lodged in some fashion in the cellular elements of the tissue. In other words, chemical mechanisms must be potentially present in the cells of living bone for (1) the solubilization of the mineral phase, (2) the destruction or solubilization of collagen, (3) the removal of polysaccharides, and (4) the destruction and removal of bone cells.

In vitro systems offer the best chance to obtain direct evidence for such local chemical mechanisms and to examine their nature and control. The success of our attempts to measure the over-all metabolism of living bone fragments *in vitro* (Borle, Nichols, and Karnovsky, 1960; Borle, Nichols, and Nichols, 1960*b*) suggested that such systems were feasible and opened a way to examine each of these four postulated mechanisms. So far, we have explored the first in some detail and have very recently begun work on the second. The last two remain untouched by us although others have found evidence suggesting their existence (Bollett, 1962; Fell *et al.*, 1962).

Our first experiments were stimulated by an interest in the mobilization of bone salts to maintain the calcium ion concentration in the extracellular fluid. At that time Neuman and collaborators (Neu-

man and Neuman, 1953) had shown that the Ca ion concentration in the extracellular fluid is higher than could be expected to exist at equilibrium between bone mineral and extracellular fluid at pH 7.4, and had proposed that bone cells produce an acid metabolite—citric acid—which not only lowers the pH locally at the bone crystal surface, but also forms a soluble complex with Ca ions, allowing them to be swept away in the circulation. Furthermore, with ingenious experiments in intact dogs (Neuman *et al.*, 1956; Firschein *et al.*, 1958) he and his coworkers produced evidence in favor of his hypothesis and showed that parathyroid hormone appeared to enhance bone citrate production. Thus, our first steps were to determine the nature and amount of acid produced by bone fragments *in vitro* and examine the influence of factors such as parathyroid hormone on the system.

Incubating fragments of cartilage-free fresh bone at 37°C in Krebs-Ringer media buffered with bicarbonate at pH 7.4, we (Borle, Nichols, and Nichols, 1960*a*) were able to show that normal bone from mice produced H^+ ion (measured manometrically by the evolution of CO_2 from the bicarbonate medium) from a glucose substrate in considerable amounts even under aerobic conditions. Moreover, acid production was increased by previous treatment of the animals with parathyroid extract. These experiments are illustrated in the left-hand part of Fig. 1. However, chemical analysis of the medium after incubation revealed that the acid anions which accumulated were almost entirely lactate, citrate accumulation being only about 1/70 as great. Two other observations of importance were made: First, both lactate and citrate production were increased by parathyroid treatment (Borle, Nichols, and Nichols, 1960*b*); and, second, acid production measured as lactate and citrate accumulation was always considerably larger than that which could be measured manometrically as free H^+ ion (Borle, Nichols, and Nichols, 1960*a*).

These experiments confirmed with direct evidence Neuman's finding of acid production by bone and its enhancement by parathyroid hormone, but indicated that lactic rather than citric acid was the chief end product. Since May 1959, when these experiments were performed, these observations have been confirmed many times

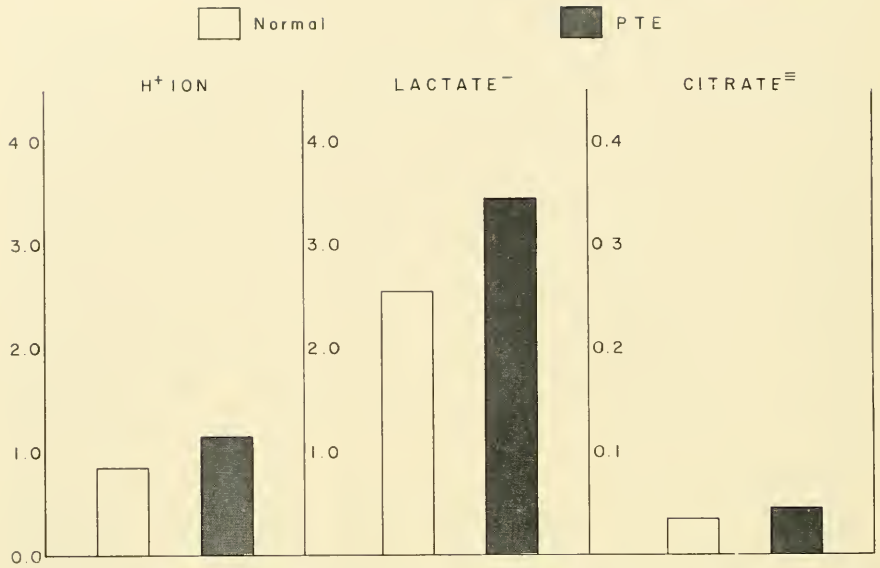

FIG. 1. The mean accumulation per hour of H^+, lactate$^-$, and citrate$^{\equiv}$ ions in the incubation medium surrounding fragments of cartilage-free metaphyseal bone from 50- to 60-day-old mice. All data have been calculated in terms of μmoles accumulated per mg of alkali-soluble nitrogen ("cell nitrogen") present in the system. Incubations were carried out for 2 hours in Krebs-Ringer media without Ca^{++} containing glucose 2 mg/ml buffered to pH 7.4 with bicarbonate under a gas phase of 5 per cent CO_2 and air at 37.5°C. H^+ ion accumulation was measured manometrically as CO_2 displaced from the incubation medium. Lactate and citrate were measured chemically. Note that the units on the ordinate for citrate$^{\equiv}$ are one-tenth those on the ordinates for the other two ions. (Data in this figure have been replotted from Borle, Nichols, and Nichols, 1960*a*, and Borle, Nichols, and Nichols, 1960*b*.)

(Dowse and Neuman, 1961; Kenny, 1961; Cohn and Forscher, 1962; Deiss *et al.*, 1962). All that remained, it seemed, was to establish the relation between acid production and Ca mobilization, and Schartum undertook to obtain the necessary evidence. Although the details of his experiments have been published (Schartum and Nichols, 1961*a*, 1961*b*), I will review them briefly.

The *in vitro* system seemed an excellent model, since Ca concentrations in the medium could be considered to bear a relation to the bone fragments similar to that which the Ca concentration in extracellular fluid bears to the skeleton *in vivo*. In preliminary ex-

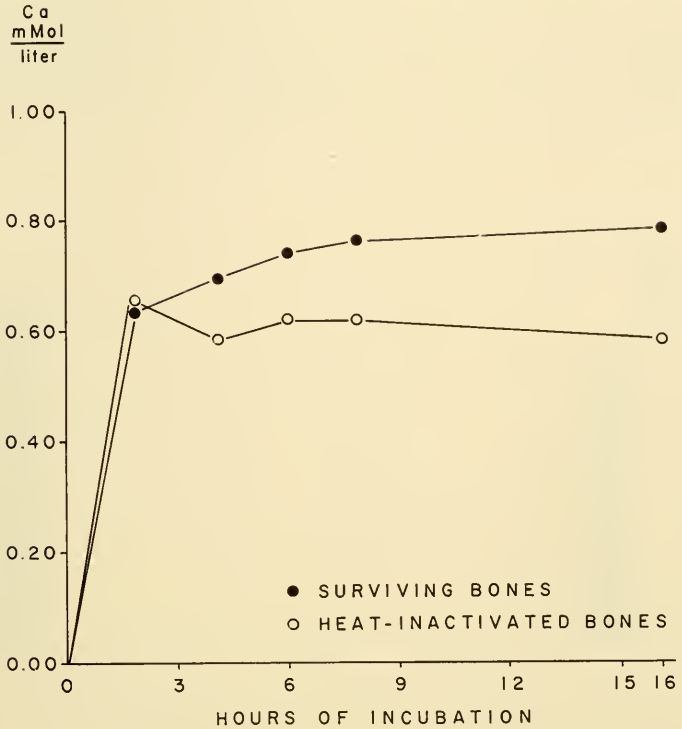

FIG. 2. Calcium concentrations found in incubation media surrounding fresh and heat-inactivated calvaria from 7- to 9-week-old mice after various periods of incubation. Krebs-Ringer media (pH 7.4) buffered with bicarbonate, a gas phase of 5 per cent CO_2 and air, and glucose 2 mg/ml as substrate were used. No calcium was present in the media at the start of incubation, but phosphorus 0.4 mmole/liter was included. Calvaria were inactivated by immersion for 1.5 minutes in medium previously brought to boiling. A "steady state" distribution of Ca and P between bone sample and incubation medium was assumed to hold after 4 hours of incubation on the basis of these findings. (This diagram has been reproduced from Schartum and Nichols, 1961*b*, with the permission of the *Journal of Clinical Investigation*.)

periments using calvaria from mice, Schartum was able to show, as indicated in Fig. 2, that the Ca concentration in incubation medium (Ca-free at the start of the experiment) rapidly reached a plateau, and the level maintained by fresh bone, which was producing acid, was 18 per cent higher than that maintained by inactivated bone which produced no acid. On the basis of this observation the total

medium Ca concentration at steady state *in vitro* could be divided into two fractions: one dependent on mineral solubility alone—the concentration maintained by inactive bone—and an additional increment dependent on the presence of cell metabolism. Moreover,

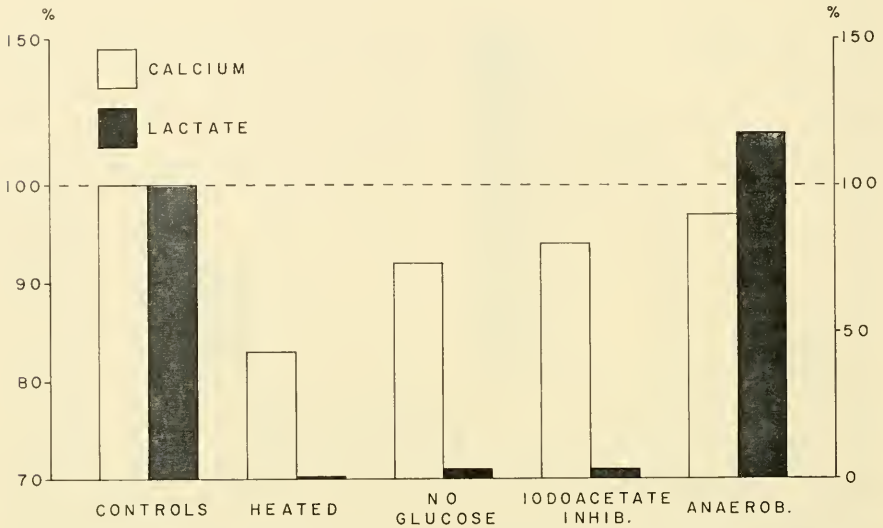

FIG. 3. Ca^{++} and lactate$^-$ concentrations in incubation media after 6 hours' incubation of calvaria from 7- to 9-week-old mice. Krebs-Ringer bicarbonate-buffered media, containing no Ca^{++} but 0.4 mmole/liter of phosphorus, and a temperature of 37.5°C were used. The gas phase was 5 per cent CO_2 in air or N_2 as appropriate. Glucose when present was at a concentration of 2 mg ml. Iodoacetate when used was added to give a final concentration of 0.9×10^{-3} M. The data in this diagram have been plotted as percentages of the values obtained in simultaneous aerobic incubations of normal samples in media containing glucose and no inhibitors. The scale for calcium is on the left, for lactate on the right. (The data shown have been recalculated from Schartum and Nichols, 1961*b*.)

metabolic acid production and this increment in Ca concentration in the media seemed at least qualitatively related.

His next series of experiments is summarized in Fig. 3. Previous experience (Borle, Nichols, and Karnovsky, 1960; Kenny, 1961) indicated that lactate and H^+ ion accumulation in the medium could be increased by incubation under N_2 and sharply reduced by the omission of glucose from the medium or by metabolic inhibitors.

Unfortunately, manipulation of lactate accumulation by these means failed to reveal any clear relationship of lactate to Ca concentration once steady state was achieved. Although, as can be seen, iodoacetate and the absence of glucose reduced lactate accumulation 90 per cent or more, Ca concentration declined only 10 per cent. Under a N_2 atmosphere lactate was increased 20 per cent, but Ca levels were if anything slightly lower than normal for fresh bone. Vaes in experiments carried on at the same time was equally unsuccessful in demonstrating any relation between citrate concentration in the medium and Ca levels (Vaes and Nichols, 1961).

Meanwhile, Raisz and his collaborators (Raisz *et al.*, 1960), using fresh bone and an analogous *in vitro* system, had shown that parathyroid treatment increased the level of Ca maintained in the medium. Schartum therefore determined to see how this parathyroid-induced increment in medium Ca concentration might be distributed between the two fractions of the total Ca concentration and whether it could be related to the changes in cell metabolism known to be produced by parathyroid extract. The results of these experiments are shown in Fig. 4. In essence, parathyroid treatment of the animals produced two effects in Schartum's system. First, the total Ca concentration maintained by fresh bone was increased (confirming Raisz's observation), but, most importantly, this effect was entirely the result of an increase in the fraction of the total which was dependent *on the solubility of the bone mineral*, the fraction related to cellular metabolism remaining essentially the same. Second, although the total fraction dependent on cell metabolism remained constant, more of it was related to carbohydrate metabolism and the availability of glucose as substrate than was true of bone from normal animals. Despite these changes in calcium, no change in phosphorus distribution was observed in any of these experiments.

These observations, we believe, have important implications regarding both the *in vitro* model system and the mechanisms involved in Ca and P distribution between the skeleton and extracellular fluids *in vivo*. In the first place, the lack of change in total medium P despite changes in Ca suggests that mechanisms are present in the skeleton which allow these two ions to be handled separately to some degree. Subsequent experiments (Nichols *et al.*, 1963;

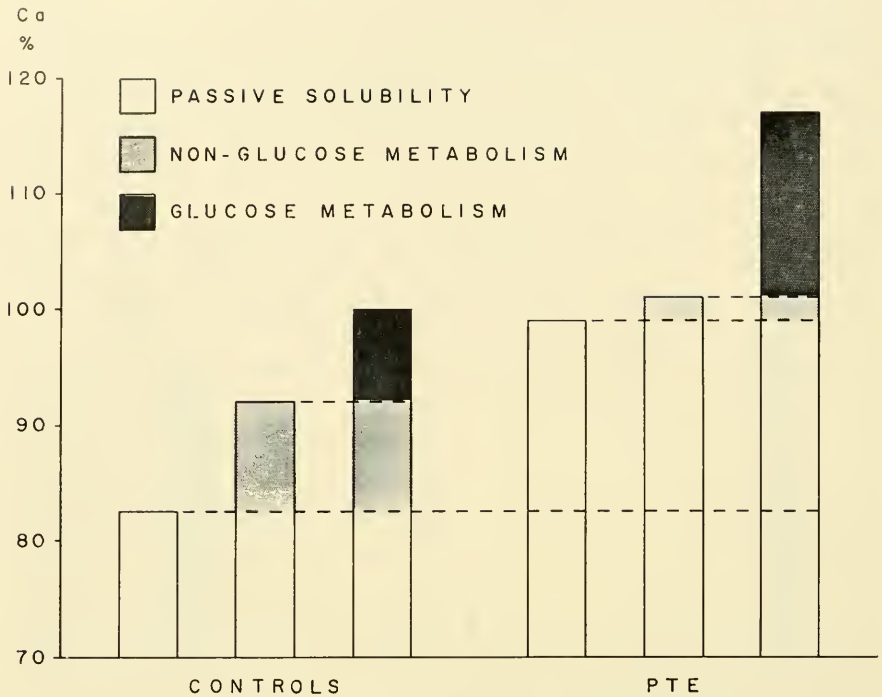

FIG. 4. Components of the "steady state" Ca^{++} concentration maintained by bone samples (calvaria) from normal and parathyroid-treated mice after 6 hours' incubation. Parathyroid-treated animals received 0.15 ml Lilly parathyroid extract subcutaneously daily for 3 days prior to sacrifice. Conditions of incubation, etc., were the same as for Figs. 2 and 3. The fraction denoted "solubility" was taken as the concentration maintained by heat-inactivated samples (cf. Fig. 2). All data are plotted as percentages of the values obtained using fresh bone from normal animals in a complete medium. (This diagram has been reproduced from Schartum and Nichols, 1961*b*, with the permission of the *Journal of Clinical Investigation*.)

Schartum and Nichols, 1961*a*) using living bone from animals treated with other agents known to affect Ca metabolism have provided further support for this view. The results of these experiments are contrasted in Fig. 5 with those of the parathyroid experiments. These data indicate that vitamin D treatment causes an increase in both Ca and P, parathyroid extract an increase in Ca only, and cortisone no change in Ca but a notable depression of P. Although not shown in the diagram, the vitamin D effects—as in the parathyroid experi-

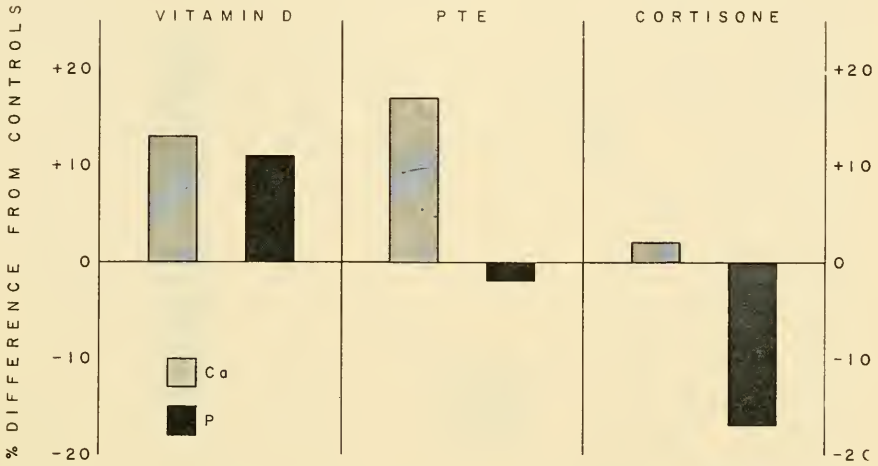

FIG. 5. Differences in final medium calcium and phosphorus concentrations from simultaneously incubated controls following treatment with vitamin D, parathyroid extract, or cortisone. Calvaria from mice 7 to 9 weeks old were used in all experiments. Animals were prepared as follows: In the vitamin D experiments, 4-week-old mice were placed on a vitamin D–free synthetic diet containing approximately 1 per cent Ca and 1 per cent P for 3 to 4 weeks. Those treated with vitamin D received a single dose of 8000 units 3 days prior to sacrifice. Animals that had received no vitamin D served as controls. The data for parathyroid extract treatment have been recalculated from Fig. 4. Cortisone acetate 2.5 mg was injected subcutaneously in normal mice daily for 8 days preceding sacrifice to give the changes seen in the last section of the diagram. Conditions of incubation, etc., were as in Figs. 2, 3, and 4. (These data have been recalculated from Schartum and Nichols, 1961*a;* Schartum and Nichols, 1961*b;* and Nichols *et al.*, 1963.)

ments—were almost entirely a reflection of change in mineral solubility.

It is of great interest that alteration in mineral solubility rather than the fraction dependent on cell metabolism seems to determine these changes in Ca and P distribution, at least as seen in this *in vitro* model. So far we have found only one case where this is not true: in a mutant strain of rats which lack the ability to resorb bone yet have normal serum Ca concentrations, low serum P, and apparently normal parathyroids (Vaes and Nichols, 1963). Figure 6 shows that the Ca level maintained by inactive bone from normal and *ia* rats, as this strain has been named (Greep, 1941), is the

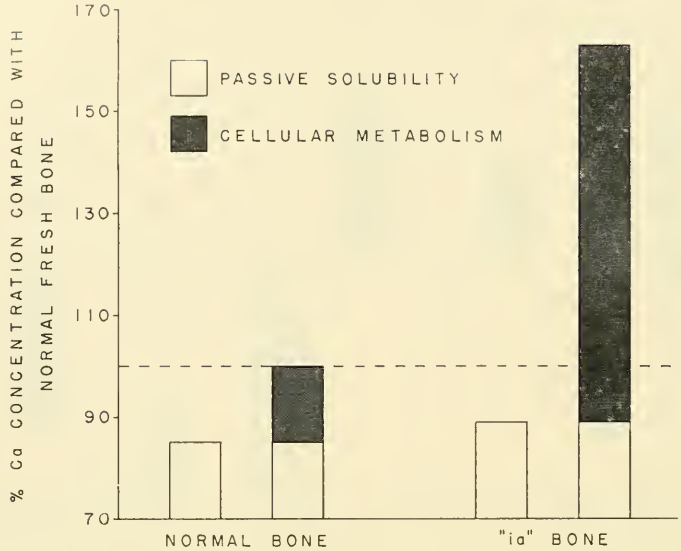

FIG. 6. Components of the total calcium concentration maintained in incubation media at "steady state" by metaphyseal bone from normal and *ia* rats 22 days old. Conditions of incubation, etc., were as in previous figures. The large increment in the fraction of the total calcium concentration dependent on cellular activity in *ia* bone can be seen to account entirely for the 60 per cent increment above controls in the total Ca concentration found in media surrounding fresh samples. (These data have been replotted from Vaes and Nichols, 1963.)

same, but the level maintained by living bone is much higher in *ia* animals. Thus, in this case, the increment in fresh bone is entirely in the fraction dependent on cellular activity. The reason for this difference between normal and *ia* rats remains unknown, but metabolic studies have shown no excessive lactate or citrate accumulation in the medium surrounding *ia* bone, although a partial defect in citrate oxidation appears to be present.

Although these experiments illustrated several important features of the distribution of Ca and P between bone and its surrounding fluids, the information needed to relate cellular metabolic activity directly to Ca and P mobilization into the medium and to changes in bone mineral solubility was still lacking. In all these experiments, however, the *in vitro* system had been viewed, naïvely, as a two-phase system, bone and medium. It became increasingly apparent

that it is, in fact, at least a three-phase system consisting of mineralized fibers covered by a very thin layer of fluid which is separated from the incubation medium by a layer of metabolically active living cells embedded in a stroma of collagen and polysaccharide ground substance, as illustrated in Fig. 7.

This view, which is consistent with the histology of the tissue, points out possible ways to explain such observations as the discrepancy between the accumulation of H^+ ion and the accumulation of organic anions in the medium. As previously noted (Fig. 1), only 1 equivalent of H^+ ion (measured by the manometric technique) was released into the medium for every 3 equivalents of lactate plus citrate. In the three-phase model the retained protons could be either retained in the layer of fluid bathing the mineralized fibers, thereby maintaining the pH of the layer at a lower level than the incubation medium, or taken up by the hydroxyapatite crystals. Moreover, the possibility was suggested that the ratio of protons and other ions retained in the sample to those released into the medium might be modified by changes in cell metabolism or the permeability of the cell layer, which might change under the influence of some of the factors studied (Borle, Nichols, and Karnovsky, 1960). Thus the three-phase model suggested possible mechanisms for the observed modification of bone mineral solubility, on the one hand, and for the influence of cell metabolism on the steady-state medium Ca concentration on the other, while pointing to possible reasons for the failure of organic anion concentration in the medium to correlate with medium Ca and P concentrations.

With these views in mind, Schartum determined to seek evidence that the pH at the mineral surface was kept at a lower level than the incubation medium by living cells and that the presence of this H^+ ion gradient was responsible for the cell-dependent fraction of the total Ca concentration in the incubation medium (Schartum and Nichols, 1962). His argument was simple: If inactivating the cells of a bone sample abolished such a H^+ ion gradient and thus lowered the steady-state Ca concentration in the medium, a similar effect should be observed if the gradient were abolished by another means in incubations using samples with actively metabolizing cells. Previous experiments (Borle *et al.*, unpublished data) had indicated

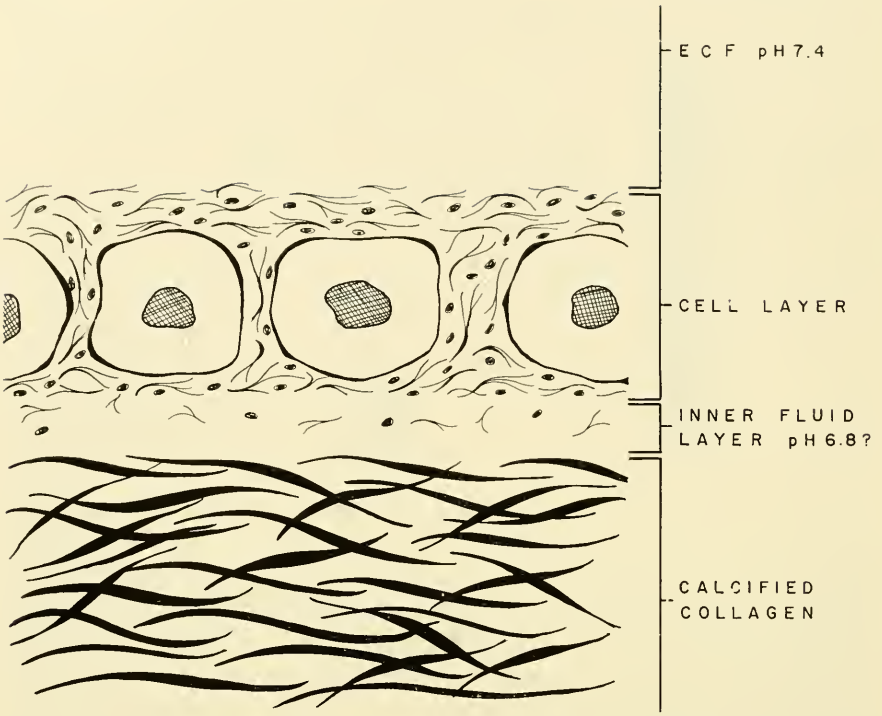

FIG. 7. A schematic view of the "interface" between the calcified collagenous matrix of bone and the circulating extracellular fluids (ECF) (see text). In the *in vitro* system described here, the incubation medium substitutes for the extracellular fluid. As conceived at present, movement of ions between calcified matrix and extracellular fluids is driven by diffusion along concentration gradients only. Such ion movement (and exchange) occurs, not directly between calcified matrix and extracellular fluid, but rather via the "inner fluid layer"; hence, a three-compartment system. The "inner fluid layer" is thought to be of very small relative volume and indeed may be partly or totally filled with an extension of the fibrillar and amorphous matrix in which the cells are embedded. Despite the ability of ions to diffuse rapidly between this inner layer and the extracellular fluids, some considerable differences in its ionic composition could be maintained under steady-state conditions thanks to its very small volume in relation to cellular metabolic activity and the high change density present in close proximity to the mineral crystals. One possible difference—in pH—is indicated in the diagram with a question mark. Some evidence in favor of this idea is shown in Fig. 8.

normal lactate production by bone cells incubated at pH's as low as 6.6. The simplest approach, therefore, was to incubate samples, both fresh and inactivated, at lower pH's and see whether the difference between the two was abolished. Figure 8 shows the results ob-

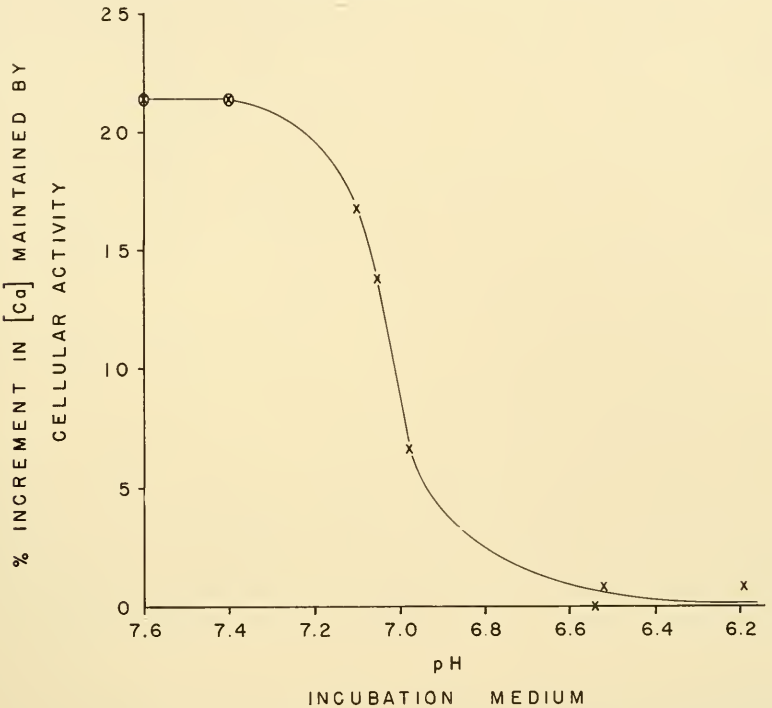

FIG. 8. The percentage increments in total calcium concentration maintained by fresh bone over heat-inactivated bone during aerobic incubation for 6 hours at 37.5°C in acetate buffers varying in pH from 7.1 to 6.2 have been plotted with data from incubations in bicarbonate-buffered media at pH 7.6 and 7.4. Each point represents the mean of 4 to 6 observations. (This diagram has been reproduced from Schartum and Nichols, 1962, where the details of these experiments are discussed. It is reproduced with the permission of the *Journal of Clinical Investigation*.)

tained. It is apparent that the *difference* in Ca concentration between fresh and inactivated samples declined very sharply below pH 7.2 and disappeared by pH 6.6, even though lactate production was unchanged—a result which fits nicely with published data on bone mineral solubility (Nordin, 1957). Thus on its first test the

three-phase model appeared to be valid, and further evidence in favor of an acid mechanism for the mobilization of Ca from the skeleton was provided.

The foregoing review of our experiments with regard to bone mineral resorption has perhaps indicated the lines which our thinking has followed and some of the difficulties we have encountered. Obviously more questions have been raised than answered, and there is patently much more to be done. It would seem, however, that such *in vitro* model systems not only are feasible but are probably sufficiently good prototypes to offer by analogy possible explanations of *in vivo* phenomena. Especially encouraging is the evidence suggesting that the bone fragment *in vitro* functions as a three-phase system, indicating that it may be an even better model of the situation *in vivo* than we had hoped.

Though the majority of our work so far has been concerned with the resorption of inorganic material, experiments have been begun to study resorption of the organic phase.

The first of these grew from evidence recently obtained by Flanagan and others that C^{14}-labeled proline from the incubation medium is incorporated into the stable collagen of fresh bone fragments during incubation (Flanagan and Nichols, 1962; Deiss *et al.*, 1962). This observation suggested a way of examining at a chemical level the notion advanced from histologic and physiologic observations (Lacroix, 1956; Jowsey, 1960; Talmage *et al.*, 1959) that bone resorption occurs at sites microscopically remote from areas of new bone formation, and at the same time possibly developing a method for studying collagen turnover time in bone. Since proline label introduced during *in vitro* incubation in labeled medium would be incorporated into the stable collagen only at sites of new bone formation, further incubation of such samples in medium containing only unlabeled proline, with examination of the collagen labeling of samples removed at various times thereafter, should indicate the biological life of the collagen deposited during the initial labeling.

Woods, who is carrying on these studies, has obtained the results shown in Fig. 9, in two typical experiments using metaphyseal bone from 50-day-old rats. The closed circles show the counts found in

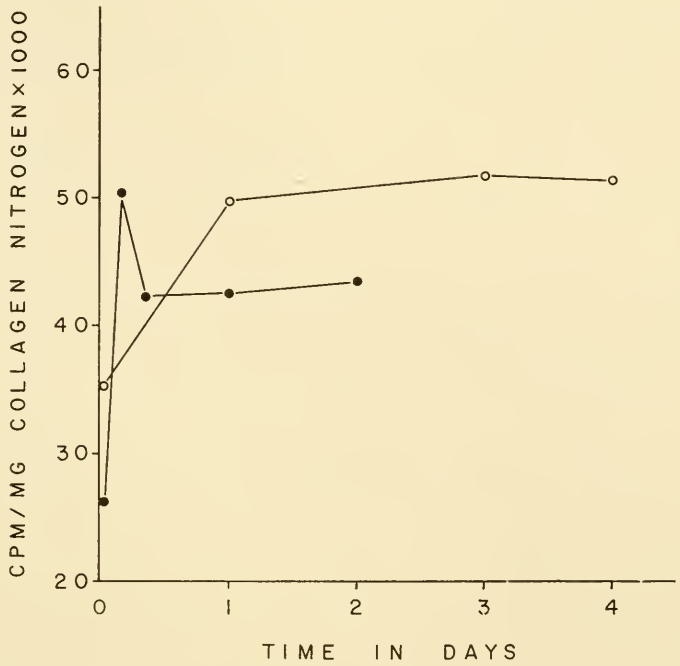

FIG. 9. Radioactivity present in the collagen of metaphyseal bone from rats at varying intervals following a 4-hour "pulse" labeling with proline-1-C^{14}. All incubations were carried out in Krebs-Ringer bicarbonate-buffered media, pH 7.4, at 37.5°C, with L-proline 0.5 mmole/liter and glucose 2 mg/ml present as substrates. Penicillin 5 u/ml and streptomycin 0.01 mg/ml were added to prevent bacterial growth.

stable collagen of bone samples at times ranging from immediately after transfer of the washed samples of bone into unlabeled media to 48 hours. It can be seen that immediately after transfer, collagen radioactivity increased, confirming Flanagan's observation of transfer of proline label from the cells of the sample into collagen. This was followed by a small abrupt decline in counts whose significance is not yet clear. From then on, however, collagen radioactivity remained quite stable save for a very slow rise. The open circles are for a longer experiment with less frequent sampling. At 4 days all the labeled collagen was still present although the cells were still metabolically active as evidenced by continued lactate production

and ability to incorporate further label if transferred back to radioactive medium. There are, however, pitfalls in this approach. In another experiment antibiotic was omitted by error from the medium, which soon developed a healthy growth of bacteria—apparently an organism capable of breaking down collagen, since radioactivity was rapidly lost and was almost totally gone by 48 hours.

Although no true bone resorption has been demonstrated in these preliminary experiments, the results offer strong corroboration to the view that bone resorption occurs at sites remote from bone formation, and that most bone collagen once laid down has a biological life of considerable duration. Moreover, it would appear likely that the cells at sites of new collagen formation are not displaced and continue synthetic activity during prolonged experiments *in vitro;* certainly they do not begin to resorb the newly formed bone during this time. The contaminated experiment further supports this view of the integrity of the synthetic sites, since when collagenolytic bacteria are present, the newly incorporated label is rapidly removed.

Finally, Woods and I have recently begun to look for evidence of the collagenolytic activity which must be present somewhere in bone. For these experiments we have used a system of macerated fresh bone incubated at 37°C under N_2 in phosphate buffers ranging in pH from 5.5 to 8. In this system the cells are intact but free swimming rather than bound in their normal anatomical sites. Since known collagenases break up collagen into small peptides rather than individual amino acids (Gallop *et al.*, 1957), we have used an increase in total ultrafiltrable hydroxyproline following incubation as an index of "collagenase-like" activity. So far only a few qualitative results have been obtained. However, ultrafiltrable peptide hydroxyproline can be shown to increase after 4 hours of incubation in this system, a phenomenon which is completely abolished by previous heating of the system to 70°C for 5 minutes. The pH optimum of this activity is still in doubt, although it seems at present to lie between pH 6 and 7. Though only of the most preliminary sort, these few observations and Gross's elegant experiments (Gross and Lapiere, 1962), which he will describe later in this symposium (chapter 26), encourage us to believe that soon much more evidence

of the presence of a collagenase in bone and perhaps something of its physiological regulation will be forthcoming.

Summary and Conclusions

It is apparent from this review that no definitive answers can yet be derived from our experience to the perplexing question of the relationship of bone cellular activity to bone resorption. Some evidence has been obtained, however, which would appear important. In the realm of mineral resorption it has been possible to show: (1) that steady-state Ca concentrations are in part dependent upon the presence of actively metabolizing cells; (2) that part but not all of the cellular effect is related to carbohydrate metabolism; (3) that increases in steady-state Ca concentrations (such as those produced by parathyroid extract or vitamin D treatment) usually are the result of increases in mineral solubility rather than in the size of the cell-dependent fraction; (4) that, nevertheless, the cell-dependent fraction of Ca concentration can be increased above normal under special circumstances; and finally (5) that Ca and P concentrations can potentially be regulated separately by mechanisms inherent in the skeleton itself. In addition, these experiments have suggested a model for interpreting results obtained in *in vitro* systems of the type used here—a model which has already proved useful in supplying corroboration of the importance of metabolic acid production to mineral mobilization.

For studies of organic matrix resorption the *in vitro* approach has also proved encouraging. Not only has it been possible to indicate more directly the separation of sites of resorption from those of new matrix synthesis, but, in addition, some evidences of collagenase activity have also begun to emerge. Moreover, Bollett (1962, personal communication) and Fell *et al.* (1962) have indicated that mechanisms for removal of polysaccharide ground substance and the bone cells themselves may be investigated in such systems.

Thus, it would appear, the tools for making the next steps toward understanding bone resorption at the chemical level are at hand. What must be done now is to exploit them with imagination and industry.

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22

In Vitro Carbohydrate Metabolism of Bone: Effect of Treatment of Intact Animal with Parathyroid Extract

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DAVID V. COHN, Kansas City Veterans Administration Hospital, Kansas City, Missouri

BONE resorption induced by parathyroid extract provides a convenient experimental system and has been used by a number of investigators in attempts to determine the chemical alterations responsible for dissolving the mineral of bone *in vivo*. Although resorption of bone has been observed to result from a variety of biologic stimuli, the effect of parathyroid extract is the simplest to produce experimentally, and it is hoped that elucidation of the mechanism of this phenomenon may provide clues for explaining resorption initiated by some of the other stimuli.

Consideration of the chemical aspects of the problem of dissolving the minerals of bone suggests three possible mechanisms. One of these would require an alteration of metabolism within the bone cells, such that an increased concentration of molecules or ions capable of chelating calcium would be produced. The second would require metabolic alteration leading to an increased concentration of hydrogen ions in the immediate environment of the bone crystals.

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The third would involve the binding of phosphate ions by conversion of inorganic phosphate to organic phosphoric esters. Only the first two of these mechanisms will be considered here, as it is thought that the third is unlikely because of the high phosphatase activity of bone.

Unless specifically stated otherwise, all the data mentioned in this review came from similar experiments. In general, parathyroid extract was administered to animals at one or more times, the animals were sacrificed, and samples of bone were taken for *in vitro* metabolic studies. Control values for comparison were obtained from similar studies of samples of bone from normal, untreated animals.

Let us consider first the possibility of a chelation mechanism. The binding of calcium by citrate is a well known phenomenon and is the basis for a theory to explain the demineralization of bone. The theory holds that an excessive production of citrate occurs locally. Although the major end product of the aerobic metabolism of glucose by slices of bone is lactate, there is some Krebs cycle activity (Cohn and Forscher, 1962*a*). The direct demonstration, in bone, of some of the Krebs cycle enzymes also has been reported (Balogh *et al.*, 1961; Kuhlman, 1960; Van Reen, 1959). An increased net production of citrate by bone in tissue culture was reported (Kenny *et al.*, 1959) when incubations were carried out in an atmosphere of 5 per cent CO_2 in oxygen, as compared with incubations in 5 per cent CO_2 in air; even in this study, however, production of lactate was 50 to 100 times greater than production of citrate. Thus, there is no question that bone cells can produce citrate, but there is some question as to whether significant concentrations of citrate accumulate and also whether parathyroid hormone influences this accumulation.

Production and oxidation of citrate by bone from normal mice and from mice treated with parathyroid hormone was studied by Vaes and Nichols (1961). They found the rate of citrate oxidation in 5 per cent CO_2 in air to be about 10 times greater than the rate of citrate production. Although treatment with parathyroid hormone increased the rate of production of citrate (when further metabolism was blocked), the rate of oxidation remained sufficiently rapid to more than handle the increased amounts of citrate.

The strongest support for the hypothesis that parathyroid hormone induces an increased accumulation of citrate in bone came from the *in vivo* work of Neuman and co-workers (Neuman *et al.*, 1956; Firschein *et al.*, 1958; Martin *et al.*, 1958) and from the *in vitro* study by Lekan *et al.* (1960). However, subsequent work has produced considerable data incompatible with this hypothesis. No evidence of an increase in the accumulation of citrate related to mineral dissolution was found either by chemical analysis (Borle *et al.*, 1960; Raisz *et al.*, 1961) or by radioisotope tracer methods (Cohn and Forscher, 1961). Although the citrate hypothesis should not be abandoned merely because of contradictory evidence, alternate possibilities should certainly be seriously considered.

The second hypothesis mentioned related to alterations in the concentration of hydrogen ions. Although the major metabolic end product in bone is an acid, lactic acid, no evidence has been found of an effect of parathyroid hormone on lactate that relates to the dissolving of minerals (Cohn and Forscher, 1962*b*; Schartum and Nichols, 1961; Raisz *et al.*, 1961). We proposed that the parathyroid hormone induces a local increase in the amount of available hydrogen ion by increasing the production of CO_2 (Cohn and Forscher, 1962*c*). The nature of this effect is shown in Table I. The data from the control slices qualitatively indicate hexose monophosphate–shunt activity, which has been verified by calculations based on lactate yields (Cohn and Forscher, 1962*a*).

TABLE I. Effect of Parathyroid Extract on Metabolism of Glucose-C^{14} in Bone

Donor animal[a]	$C^{14}O_2$ (cpm)		
	Glucose-1-C^{14}	Glucose-6-C^{14}	Glucose-U-C^{14}
Control	17,200	3,900	11,100
PTE treated	23,300	6,700	16,000
Per cent increase	35	72	45
Control	27,300	6,100	17,600
PTE treated	32,100	11,300	29,300
Per cent increase	18	85	66

[a] Rabbit.

There is an important relationship between the relative magnitude of the increase in $C^{14}O_2$ and the position of the label in the glucose molecule. It shows that we are dealing with a specific metabolic alteration rather than a generalized increase in cellular metabolic activity or an increase in the number of metabolizing cells. Increased production of CO_2 can also be demonstrated with pyruvate-2-C^{14} as the substrate (Cohn and Forscher, 1961).

The differential effect shown in Table I could be the result of one of two possible types of alteration. The data would be consistent with (1) an increased flow of metabolites through the Embden-Meyerhof and the Krebs pathways with no change in the rate of operation of the hexose monophosphate shunt, or with (2) an increased rate of operation of some pathway involving preferential oxidation of the 6-carbon of glucose. When incubations are carried out in the presence of fluoroacetate, as shown in Table II, the in-

TABLE II. Effect of Fluoroacetate on Metabolism in Bone

	End products					
	$C^{14}O_2$ (cpm)		Citrate-C^{14} (cpm)		Lactate (μmoles)	
Donor animal	Substrate[a]	Substrate +FA[b]	Substrate	Substrate +FA	Substrate	Substrate +FA
Control	4,300	650	630	14,000	4.0	3.8
PTE treated	5,400	610	630	10,300	4.1	3.8

[a] Each flask contained 10 μmoles glucose, labeled with 2×10^6 cpm glucose-6-C^{14}, in 9.5 ml of Krebs bicarbonate buffer.

[b] Fluoroacetate, 50 μmoles added to solution of substrate.

creased CO_2 production due to pretreatment with parathyroid hormone disappears, indicating that the extra CO_2 from C-6 of glucose originates in the Krebs cycle. This observation correlates with that of Raisz and co-workers (1961) that in the presence of fluoroacetate there was decreased mobilization of calcium into the medium despite an increased accumulation of citrate. They also found that iodoacetate decreased the mobilization of calcium, in agreement with Schartum and Nichols (1961). Thus the metabolic activity respon-

sible for dissolving the minerals is in the Embden-Meyerhof-Krebs system, and the hormone effect also is exerted in this system.

There are several apparent contradictions to our carbonic acid theory that must be resolved. Vaes and Nichols (1962) used glucose-U-C^{14} as a substrate and could find no change in the CO_2. However, they used a substrate concentration of 11.1 mM, and we have found (Cohn and Forscher, 1962*b*) that in bone the CO_2 effect is sensitive to the concentration of the substrate. We have demonstrated this for glucose-U-C^{14} as well as for glucose-6-C^{14}. With the uniform label, the effect could not be detected when the concentration of the substrate was 4 mM or more. Using manometric methods, Laskin and Engel (1956) studied the effects of pretreatment with parathyroid extract on the metabolic activity of slices of bone and did not detect the changes in the production of CO_2 which we describe as evidence of the basic action of the hormone. We also did not find these changes when we used a Warburg respirometer; as Laskin and Engel reported, we found no evidence of utilization of exogenous glucose with such incubations. In fact, were it not for the greater sensitivity of radioisotope tracer methods, one might conclude that there was no utilization of exogenous glucose. From the appearance of C^{14} in the CO_2, in lactate, and in other intermediates, we know that the added substrate is metabolized.

Our hypothesis is that excess parathyroid hormone causes an increased production of CO_2 from glucose or from metabolites derived from glucose. Calculations based on the specific activity of the added glucose and on the radioactivity of the CO_2 produced show that the difference between the production of CO_2 in the control and in the experimental flasks would be about 0.2 μl per hour, an increment which is not detectable in the standard Warburg respirometer. The apparent failure of *in vitro* slice preparations to use an exogenous substrate is known (Robinson, 1949), and we make the assumption that, in the body, exogenous glucose is a major substrate for cellular metabolism.

Two alternate explanations of our data must be settled. One possibility is that the metabolic effect we observed was due to a general or random influence of the hormone and was not necessarily related

to its specific physiologic action. A second possibility is that the metabolic effect was secondary to the abnormally high concentration of serum calcium associated with treatment with the parathyroid hormone. To rule out the first possibility, we performed experiments similar to those performed with slices of bone, but used instead slices of kidney, liver, and muscle. Kidney is also a target organ of parathyroid hormone, and results were similar to those obtained with bone (Table III). Other tissues from the same animals, such as liver and muscle, did not produce the CO_2 effect (Table III).

TABLE III. Effect of Parathyroid Extract on Production of $C^{14}O_2$ by Kidney and Other Tissues

		Glucose		$C^{14}O_2$		
Experiment	Tissue	Position of label	μmoles	Control (cpm)	PTE (cpm)	Per cent change
A	Bone	C-1	10	4,200	5,700	36
	Kidney	C-1	10	18,900	23,400	24
	Liver	C-1	10	4,300	3,700	−14
B	Liver	C-6	10	1,850	1,400	−24
	Heart	C-6	10	750	410	−45
	Muscle	C-6	10	90	70	−22
C	Kidney	C-1	10	10,500	13,600	30
		C-6	10	6,200	11,200	81
D	Kidney	C-6	3	49,700	90,000	82
		C-6	24	13,700	26,200	91

The second possibility was examined in two ways: by experimenting with rabbits whose concentration of serum calcium had been artificially maintained at a high level, and by experimenting with parathyroidectomized rats.

In the first of these studies an increased concentration of serum calcium was maintained in rabbits with subcutaneous injections of a neutral suspension of calcium chloride in olive oil. After 6 hours, these animals and their controls were sacrificed. *In vitro* metabolic studies were then carried out on samples of bone and kidney. The data (Table IV) show two important findings. One is that the high calcium levels in the serum do not affect the metabolic pattern of

TABLE IV. Effect of Serum Calcium Concentration on Production of $C^{14}O_2$

		$C^{14}O_2$ (cpm)			
		Bone		Kidney	
Experiment	Substrate	Control	Increased calcium[a]	Control	Increased calcium[a]
I	Glucose-U-C^{14}	15,060	14,870 (−1.3%)	52,010	77,510 (+49%)
II	Glucose-6-C^{14}	6,180	5,080 (−18%)	37,470	67,310 (+80%)
	Glucose-1-C^{14}	—	—	55,710	97,030 (+74%)

[a] Mean serum calcium 8 mEq/liter more than in control.

the bone. The other finding of significance is that of the effect of the calcium treatment on the kidney. In this tissue there was an increase in CO_2 production, but of a nonspecific nature, since there was no differential between carbons of position 1 and position 6 of glucose. This further indicates that the differential after treatment with the hormone resulted from a metabolic alteration.

The second study to rule out the effect of high concentrations of serum calcium was carried out on parathyroidectomized rats. $C^{14}O_2$ production from glucose-6-C^{14} by slices of rat bone *in vitro* was not influenced by pretreatment of normal animals with parathyroid extract. However, $C^{14}O_2$ production was reduced with bone from parathyroidectomized donors. It was normal with bone from such rats treated with parathyroid extract (Table V).

It is important to consider whether or not the increased CO_2

TABLE V. Effect of Parathyroidectomy on Production of $C^{14}O_2$ by Bone

Donor animal[a]	Pretreatment	$C^{14}O_2$ (cpm)[b]
Normal	None	17,000
	200 units PTE	16,000
Parathyroidectomized	None	12,000
	200 units PTE	16,000

[a] Rat.
[b] Substrate, glucose-6-C^{14}.

production that we have described could be responsible for dissolving the minerals in bone. We have no data concerning this aspect of the problem and must rely on reasoning and on evidence from other studies. From the increased radioactivity of the CO_2, when the substrate was uniformly labeled glucose, we estimated the *in vivo* effect of the parathyroid hormone to result in a 50 per cent increase in production of CO_2. Using the Henderson-Hasselbach equation, and assuming the initial ratio of salt anion to undissociated acid to be about the same as that in blood, that is, 20 to 1 for the bicarbonate–carbonic acid system, this increase in CO_2 would be equivalent to decreasing the pH about 0.2 unit. The significance of such a change in pH would lie not in its magnitude so much as in its persistence. It has been suggested that the solubility of minerals of bone is very sensitive to change in pH in the region of pH 7 (Neuman and Neuman, 1958). Even if the absolute effect on solubility were small, living bone constitutes an open-end system in equilibrium with the blood, and a shift in the equilibrium position in the direction of the solution phase would mean a continuous drain of mineral components into the blood. This shift would be maintained as long as the altered metabolic pattern in the bone cells remained in effect. Such a shift to the solution phase has been demonstrated in *in vitro* incubations of slices of bone from animals previously treated with parathyroid hormone (Vaes and Nichols, 1962; Raisz *et al.*, 1961; Schartum and Nichols, 1961; and others).

If the effect of parathyroid hormone on bone is due to carbonic acid, could one demonstrate this by detecting a change in local pH? Probably not. In fact, two recent reports (Raisz *et al.*, 1961; Schartum and Nichols, 1961) indicate specifically that in the *in vitro* incubations studied, the increased concentrations of calcium that developed in the medium around slices of bone from animals treated with parathyroid hormone were not accompanied by related changes in pH. The reason for this is that the bone mineral acts as a buffer by dissolving in response to an increased local production of hydrogen ions. It has been suggested (Schartum and Nichols, 1962) that pH changes are involved by virtue of a pH gradient between a fluid phase in bone and the circulating fluids.

If the mechanism of action of a hormone on one tissue is due to a specific metabolic alteration, and if that same hormone has another physiologic action on another tissue, we should make an attempt to explain the second action in terms of the same biochemical mechanism that was suggested for the first action. The effect of the parathyroid hormone on the kidney is related to the excretion of phosphate. Without going into the details of the renal handling of phosphate, we might suggest that possibly the ionic form of the phosphate is important. An increased local production of carbonic acid would alter the ratios of the different forms of phosphate to one another; the major change would be a shift from the monohydrogen form to the dihydrogen form.

The bulk of present evidence is in agreement concerning the necessity of cellular metabolism for dissolving minerals of bone. Much of this evidence suggests that parathyroid hormone increases the rate of some part or parts of cellular metabolism in bone. It seems more reasonable that the effect of the hormone on the structure of bone is mediated by an end product of this metabolism than by an intermediate product in the metabolic process. However, the need for active metabolism does not necessarily mean that the process of dissolution requires energy.

It appears that the activity of the hexose monophosphate shunt is not related to the effect of the hormone, so we shall concentrate on the glycolytic and Krebs pathways. The marked accumulation of lactate, even under aerobic conditions, suggests that the limiting step in the sequence occurs after pyruvate. Since we found that the parathyroid hormone increases CO_2 production with pyruvate-2-C^{14} as the substrate but not with citrate-1,5-C^{14} (Cohn and Forscher, 1961), and with lactate-1-C^{14} as substrate but not with succinate-1,4-C^{14} (Cohn, 1962), we might narrow our attention to the sequence between pyruvate and citrate. Raisz and co-workers (1961) also did not find an effect on CO_2 labeling with acetate-1-C^{14} as substrate; but, in their experiments, glucose was present in high concentrations.

The CO_2 production relevant to the effect of parathyroid hormone requires the operation of the Krebs cycle. We suggest that the rate of operation of the Krebs cycle is controlled by the entry of me-

tabolites through the pyruvic acid–oxidase complex to acetyl coenzyme A and through the condensing system or "citrogenase." Excess parathyroid hormone increases the flow through this bottleneck (Table VI). This opening of a bottleneck is suggested not only by

TABLE VI. SUMMARY[a] OF EFFECT OF PARATHYROID EXTRACT ON PRODUCTION OF $C^{14}O_2$ BY BONE

Substrate	$C^{14}O_2$ (cpm)		
	Control	PTE	Per cent change
Glucose-U-C^{14}	11,100	16,000	44
Glucose-1-C^{14}	17,200	23,300	35
Glucose-6-C^{14}	6,390	10,320	62
Pyruvate-2-C^{14}	52,000	65,800	27
Lactate-1-C^{14}	27,870	40,240	44
Citrate-1,5-C^{14}	3,900	4,300	N.S.
Succinate-1,4-C^{14}	16,900	10,300	−39

[a] Taken from different experiments with slightly different experimental conditions. However, for each substrate, conditions were identical for tissues derived from control animals and from animals treated with parathyroid extract.

increased amounts of $C^{14}O_2$ from C^{14}-labeled precursors before the bottleneck, but also by the decreased amounts of $C^{14}O_2$ from precursors after the bottleneck, which decrease presumably results from dilution secondary to increased flow. There is no real accumulation of metabolic citrate, since the reactions of the Krebs cycle follow in sequence. If more citrate is produced in the cell, more is oxodized. The real consequence of increasing the flow through the bottleneck is a greater production of CO_2.

We do not yet have sufficient data to establish clearly which of the two enzyme systems between pyruvate and citrate is the actual controlling factor. At this time we might tentatively postulate that the crucial point in the sequence, and the site of hormone action, is in the pyruvic acid–oxidase complex. This suggestion derives from the fact that lactate, which accumulates in large amounts, is the major end product. If the conversion of pyruvate to acetyl coenzyme A proceeded rapidly, and the condensation to citrate were the limiting reaction, acetate or some other derivative of acetyl coenzyme A should be the accumulating end product.

Summary

We propose that an excess of parathyroid hormone induces an increased production of CO_2 from glucose or metabolites of glucose by cells in bone. This theory is in accord with data from many independent studies. Of the various hypotheses posed to explain the mechanism of action of parathyroid hormone, this concept explains the present evidence in a more satisfactory manner than theories based on the accumulation of citrate or lactic acid.

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23

Metabolic Action of Parathyroid Hormone on Rat Calvaria

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DESPITE a long history of polemical argument, it is now generally agreed that parathyroid hormone has a direct action on bone, causing an increase in the population of resorbing cells, the osteoclasts, a mobilization of bone mineral, and perhaps a general increase in the availability of the bone mineral, at least for exchange with parenterally administered isotopes (Greep and Talmage, 1961; Neuman and Neuman, 1958). More recently, attention has focused on possible hormonally induced changes in cellular metabolism in bone. Presumably an understanding of these metabolic changes might clarify the biochemical mechanisms by which the hormone exerts its osteolytic effects.

Bone, however, is so obviously unsuited for conventional biochemical studies of metabolism that progress has been rather limited and published reports have been few (Laskin and Engel, 1956; Borle *et al.*, 1960*a*, 1960*b*; Lekan *et al.*, 1960; Neuman and Dowse, 1961; Cohn and Forscher, 1961, 1962*a*, 1962*b*; Vaes and Nichols, 1961, 1962; Schartum and Nichols, 1961, 1962). In most cases crushed or sliced metaphyses have been employed despite the obviously serious contamination by marrow cells. The investigations reported below were conducted on the calvarium of the newborn rat.

Calvaria have been extensively employed in a variety of studies designed to show histological effects of parathyroid hormone (Gaillard, 1959), resorption in surviving culture (Goldhaber, 1960), and the cellular role in maintaining calcium levels *above* those due to passive solubility only (Raisz *et al.*, 1961). However, no comprehensive biochemical studies of calvaria have to our knowledge yet appeared. This is a ready-made tissue slice with a minimum of cut or damaged cells. It has some mineralized matrix, but the cell population, for bone, is high. There is but little contamination with marrow cells. Morphologically, however, at least five general cell types predominate: osteoblasts, osteocytes, osteoclasts, cartilage cells, and preosseous mesenchymal cells. The principal advantages of this tissue are convenience, reproducibility, and responsiveness to parathyroid hormone *in vitro* (Gaillard, 1959). Nonetheless, the calvarium still represents something of a compromise, and it is therefore emphasized that the studies to be reported are on the metabolism of calvarium. Though the data *may* apply to bone cells in general, no such claim is made.

Experimental

Procedure

Randomized newborn rat pups were decapitated within 15 hours of birth. The calvarium, a round section from the top of the skull, including most of the parietal and parts of the frontal and occipital bones, was quickly removed, freed of bits of connective tissue, and halved at the midline. Left and right halves were placed alternately in each of two weighed collecting bottles containing ice cold incubation medium. Wet weight was used as the basis of reference, since it was found at least as reliable as dry weight, noncollagen nitrogen (NCN), or deoxyribonucleic acid (DNA).

The medium employed was either Krebs-Ringer phosphate or bicarbonate with the calcium reduced to a level of 1.2 mM or absent. Incubations were performed in a Warburg apparatus at 37°C after equilibration with the appropriate gas. Control and experimental measurements were made on paired flasks containing 6 to 10 alternating halves of the same calvaria.

Methods

Standard methods were employed for calcium (Chen and Toribara, 1953), PO_4 (Chen *et al.*, 1956), lactate (Barker and Summerson, 1941), citrate (Ettinger *et al.*, 1952), carbonate (Umbreit *et al.*, 1959), NCN (Lilienthal *et al.*, 1950), DNA (Leslie and Davidson, 1951), glucose (Saifer and Gerstenfeld, 1958), and glycogen after alcohol precipitation (Umbreit *et al.*, 1959).

The calvarium was selected for study because it was presumed that its cell population would consist primarily of osteocytes and osteoblasts. Histological examination of the tissue, however, showed a significant number of preosseous mesenchymal cells. How much of the characteristics of osteoblasts the latter have assumed is undeterminable.

TABLE I. General Analytical Findings on Calvaria
(All concentrations given in terms of wet weight)

Average wet weight	15.0 ± 0.2 mg
Wet wt/dry wt ratio	4.63 ± 0.05
Calcium	33.8 ± 0.6 mg/gm
Phosphorus	17.9 ± 0.2 mg/gm
Ca/P mole ratio	1.47 ± 0.05
Citrate	1.08 ± 0.08 mg/gm
Carbonate (as CO_2)	3.7 ± 0.1 mg/gm
DNA	1.78 ± 0.03 mg/gm
NCN	9.2 ± 0.1 mg/gm

Table I summarizes some of the tissue analyses performed. The values are given plus or minus the standard error of the mean, and represent at least 25 analyses. They indicate that, chemically, the tissue is intermediate between soft tissue and mature bone.

DNA and NCN were at first considered as possible bases for metabolic reference, but the constancy of the ratio they bore to wet weight rendered these measurements unnecessary. Further, in the case of NCN, the finding that no less than 50 per cent of the NCN originally present in the tissue passed into the medium during incubation made this parameter, in our view, of questionable value.

It will be seen that the oxidative and glycolytic properties of this

tissue are distinctly different from those of most soft tissue and must be representative, in a qualitative way, of new bone or bone-forming tissue.

Results and Discussion

Because of the many metabolic aspects to be covered, a discussion of each is presented along with the results.

Oxidation

The rate of oxygen consumption of calvaria in phosphate medium containing no added substrate is presented in Fig. 1A. Despite the

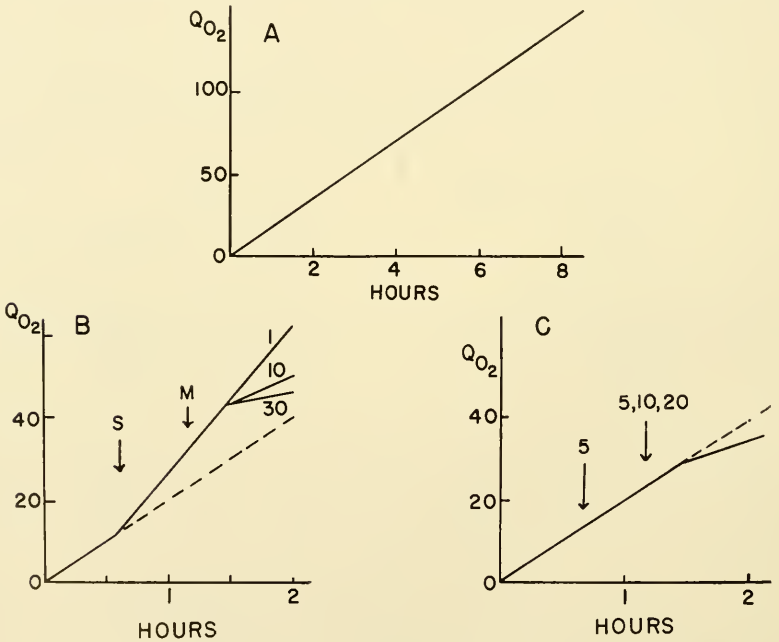

Fig. 1. Oxygen consumption of calvaria incubated in phosphate medium *in vitro*. Q_{O_2} expressed as μmoles per gm wet weight. A, data showing the continued linear oxygen consumption over an 8-hour period. B, data showing the enhanced oxygen consumption from the addition of 10 mM succinate (*S*) and subsequent inhibition by malonate (*M*); numbers indicate concentration in mM. C, data showing the effects of additions of the inhibitor fluoroacetate; numbers indicate its concentration in mM.

constancy of the rate over an incubation period of 8 hours, all subsequent studies were conducted for 3 hours or less. The average Q_{O_2} of 10 different experiments, 18.4 ± 2.5 μmoles/gm wet wt/hr., is intermediate between earlier values reported for metaphysis and for a truly active soft tissue such as liver:

	Q_{O_2} μmoles/mg NCN	Q_{O_2} μmoles/mg DNA
Metaphysis (Borle *et al.*, 1960*a*, 1960*b*)	1.1	
Metaphysis (Laskin and Engel, 1956)		57
Calvarium	2.0	230
Liver (Spector, 1956)		850

No effect on the Q_{O_2} could be found upon addition of a variety of substrates, including glucose, pyruvate, acetate, fumarate, malate, oxaloacetate, citrate, and alpha ketoglutarate either singly or in various combinations best calculated to stimulate oxygen uptake. Similar findings were reported by Laskin and Engel using metaphyseal preparations (Laskin and Engel, 1956).

Succinate was the only substrate found to have any effect (Fig. 1B): a 50 per cent increase at a concentration of 10 mM or higher. This stimulation was abolished by the subsequent addition of malonate at an equal or higher concentration. Note that, whereas the succinate effect is immediate, the malonate effect takes some 15 to 20 minutes to develop. Because of its ability to participate in substrate-linked oxidative phosphorylation independent of the pyridine nucleotides, however, succinate occupies a special place in the Krebs (TCA) cycle, and stimulation of oxygen uptake in a tissue upon its addition is *not* evidence of increased TCA cycle activity, but only of the availability of an excess capacity in the succinic dehydrogenase–flavoprotein system. Malonate does more than abolish the increase due to succinate. It produces, in turn, a further reduction in the Q_{O_2} which, relatively to the endogenous rate, is equal to that produced by malonate additions alone (not shown). In experiments both with and without succinate, a concentration effect of malonate was noted: 1 mM not affecting the Q_{O_2}, 30 mM reducing it by some 25 to 30 per cent, 10 mM being intermediate. The con-

centration dependency of the effect is to be expected for a competitive inhibitor of this type. Addition of fumarate, after malonate, at levels up to twice that of the malonate concentration used, did not relieve the block, nor did addition of oxaloacetate. The latter was of interest because malonic acid has been shown to be an inhibitor of fumarase as well as of succinic dehydrogenase (Massey, 1953). It would appear, therefore, that malonic acid was eliminating, by a competitive inhibition of succinic dehydrogenase, O_2 uptake due to succinate oxidation, but was *not* reducing the availability of dicarboxylic acids for the functioning of the rest of the cycle.

A single addition of fluoroacetate at the 5-mM level had no effect on endogenous Q_{O_2} (Fig. 1C), whereas a second addition at the same concentration, or single additions at the 10- or 20-mM level, reduced the Q_{O_2} by some 25 per cent. There was no greater effect at the highest concentration. This, as well as the delay period before the effect became manifest, is consistent with the noncompetitive nature of the inhibition, and with the necessity for prior incorporation of the fluoroacetate into the TCA cycle to form fluorocitric acid. The continued oxygen uptake, with fluoroacetate at the 20-mM level, could be due to an incomplete block at the aconitase stage, perhaps because of an adequate supply of endogenous pyruvate. If, instead of being added during the incubation, 10-mM fluoroacetate was present from the beginning, the reduction in Q_{O_2} could be prevented by the coincident addition of pyruvate at 10 mM, but not of fumarate at 10 mM or glucose at 17 mM (Table II). This protective property

TABLE II. The Effects of Fluoroacetate and Substrates on Oxygen Consumption

(Phosphate medium; 100 per cent O_2, 2-hour incubation; averages of 4 to 8 flasks)

Additions	Q_{O_2} (μmoles/gm wet wt/hr.)			
	Fluoroacetate 10 mM		No fluoroacetate	
	No glucose	Glucose 17 mM	No glucose	Glucose 17 mM
None	11.6	10.7	17.4	17.4
Fumarate 10 mM	9.8	11.2	17.4	17.0
Pyruvate 10 mM	18.2	17.4	17.8	17.4
Fumarate 10 mM : pyruvate 10 mM	18.2	17.8	17.8	17.0

of pyruvate is most satisfactorily explained in terms of a competition between pyruvate and fluoroacetate for coenzyme A, for it is well known that if pyruvate and fluoroacetate are present together, the greater amount of carbon incorporated into the cycle is derived from the pyruvate.

As a consequence of its inhibitor action upon aconitase, fluoroacetate poisoning of a tissue with a functioning TCA cycle usually results in the accumulation of citric acid. Because of the large quantity of citric acid which is present in bone mineral, however, increased citrate concentration in response to fluoroacetate injection *in vivo* has not been shown to occur (Beaulieu, 1953). In a closed system *in vitro,* changes in citrate content are easily demonstrable.

Analyses of the medium and tissue showed that glucose addition did not increase citrate accumulation, whereas, as shown in Table III, fumarate on the one hand and fluoroacetate on the other, alone

TABLE III. The Effect of Fluoroacetate and Substrates on Citrate[a] and Lactate Production

(Bicarbonate medium; glucose 17 mM; fumarate 5 mM; fluoroacetate 5 mM; 2-hour incubation; averages of 4 to 8 flasks)

	Citric acid (μmoles/gm wet wt)			Lactic acid production (μmoles/gm wet wt)
	Medium	Tissue	Total	
Glucose	0.9	3.8	4.7	30.4
Glucose : fumarate	1.7	4.6	6.3	31.3
Glucose : fluoroacetate	1.2	5.2	6.4	29.8
Glucose : fumarate : fluoroacetate	2.6	5.1	7.7	30.2
Original tissue content			5.6	0.2

[a] Note that with glucose only, citrate is actually *utilized* despite its appearance in the medium. Because of ease of exchange of citrate between tissue and medium it is necessary to analyze the system *in toto.*

or in combination, did bring about an increase in total citrate. Even with fumarate and fluoroacetate present at the same time, the rate of appearance of citric acid in the medium was very considerably less (15 times) than that of lactate. Lactic acid production was not significantly affected by the various additions, except, of course, by the addition of glucose.

There would seem, considering all the fragmentary data collected here and elsewhere (Cohn and Forscher, 1962*a*, 1962*b*; Lekan *et al.*, 1960), to be no reason to doubt the presence of the TCA cycle in bone. In the calvarium it is present from succinate to citrate, and citrate can be further metabolized. The failure to show effects on the Q_{O_2} by addition of intermediates is not really surprising, for it is necessary to deplete the endogenous supply of substrate in a tissue before this can be achieved. Subsequent addition of the intermediate, then, merely results in a return of the Q_{O_2} to the initial value. There is also, in the data presented, no reason to conclude that the oxygen uptake results from systems other than the TCA cycle, for in a tissue exhibiting a continuing aerobic glycolysis (see below) the availability, of pyruvate at least, for the TCA cycle will not be limiting.

Glycolysis

Like other "bone" preparations (Cohn and Forscher, 1962*b*; Laskin and Engel, 1956; Borle *et al.*, 1960*a*, 1960*b*), calvaria show high rates of glycolysis even under aerobic conditions. With no added substrate, glycolysis (defined as lactate production) decreases with time; with added glucose, the rate is linear for at least 3 hours (Fig. 2A). Anaerobically, the initial glycolytic rates are 2 to 2.5 times higher. Though added glucose maintains the initial rate for 3 hours, in the absence of added substrate the rate falls off even more rapidly than it does aerobically. Thus, after 2 or 3 hours' incubation total lactate accumulation is about the same, anaerobically or aerobically. This may account for the failure of some investigators to observe the Pasteur effect (Lekan *et al.*, 1960).

When shorter periods of incubation are employed, the effect of oxygen on endogenous glycolysis is readily apparent, as seen in Table IV. Here periods of 30 and 60 minutes were used, and a Pasteur effect of some 70 per cent was found.

Lactate production increases with increasing concentrations of glucose up to about 4 mM (Fig. 2B). Thereafter the Q_L increases less rapidly, especially in the bicarbonate medium.

A marked effect of the buffer present, seen in Fig. 2B, is further demonstrated in Table IV. In the bicarbonate-buffered medium, the

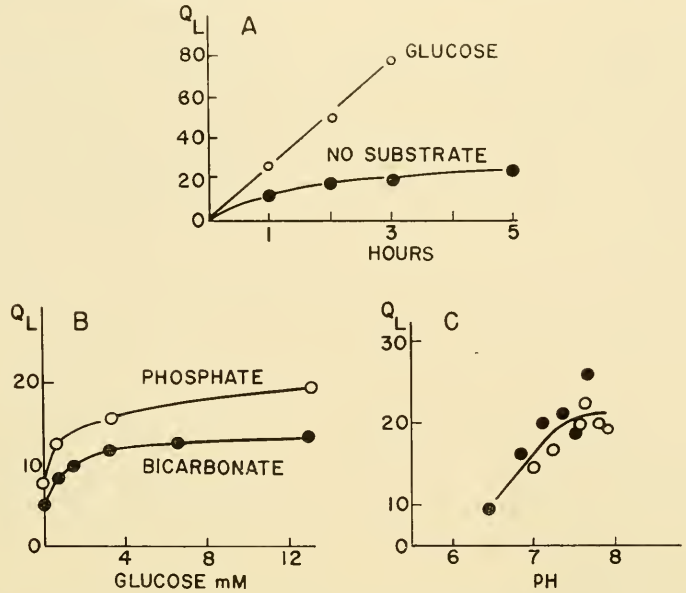

FIG. 2. Lactate production by calvaria incubated *in vitro*. A, the Q_L in μmoles per gm wet weight with time in the presence and absence of added glucose. B, the variation in Q_L as μmoles/gm/hr. with varying glucose concentration in both phosphate and bicarbonate media. C, the variation in Q_L with variation in pH; phosphate medium, glucose 17 mM.

bicarbonate ion concentration was 25 mM, phosphate 1.2 mM; in the phosphate medium, phosphate was 16 mM, bicarbonate being absent. Interestingly enough, the medium effect was missing under anaerobic conditions (Table IV), lactate production in both cases

TABLE IV. THE EFFECT OF MEDIUM ON LACTATE PRODUCTION
(Mean values of six flasks)

	Lactic acid production (μmoles/gm wet wt)			
	30-minute incubation		60-minute incubation	
Medium	Aerobic	Anaerobic	Aerobic	Anaerobic
Bicarbonate	3.2	10.8	5.8	17.1
Phosphate	5.0	10.9	7.5	16.5

being approximately twice that in the phosphate buffer aerobically.

It is difficult to find an adequate explanation for effects of this kind involving phosphate and bicarbonate; effects which are not confined to bone cells. Several possibilities, however, come to mind. One involves the intracellular level of inorganic phosphate, on the assumption that inorganic phosphate is one of the regulatory factors of the glycolytic rate. Thus, in ascites tumor cells, glycolysis can be markedly increased by increasing the concentration of inorganic phosphate in the medium and thereby, indirectly, in the cell (Racker and Wu, 1959). However, though increase in phosphate ion concentration to levels of 40 mM led to *some* further stimulation of glycolysis, it was not very great, and not of the order obtained with ascites preparations. Also, addition of bicarbonate suppressed the phosphate effect. Thus, in a medium containing both phosphate at 16-mM and bicarbonate at 25-mM concentrations the Q_L was almost identical with the quantity found in the standard bicarbonate-buffered medium.

We have been unable to find a difference between Q_{O_2} in bicarbonate-buffered medium on the one hand and phosphate-buffered medium on the other using the CO_2 buffer technique suggested by Pardee and developed in detail by Krebs (Umbreit *et al.*, 1959). If Warburg's "indirect" method is used, however, the Q_{O_2} appears to be increased some threefold in the presence of bicarbonate. We can suggest no explanation for the lack of agreement between the results obtained with the two methods, though a variable CO_2 retention by the tissue, as postulated by Borle (Borle *et al.*, 1960*a*, 1960*b*), is one possibility. Until this problem can be resolved, the possible effect of different oxidation rates upon glycolysis in the two media cannot be determined.

Another explanation involves control of the intracellular pH. One would expect extracellular bicarbonate to exert a more efficient control over the intracellular pH than phosphate, because of the difference in permeability characteristics of the two ions. However, the crucial experiment has not been devised to test this hypothesis. Lactate production is only slightly affected by changes in the pH in phosphate medium between 7.3 and 7.8; below this pH, Q_L is decreased (Fig. 2C).

Substrate Utilization

The Q_{O_2} remains stable for many hours with no added substrate. From this, it is obvious that an adequate supply of the intermediates and substrates exists for the system or systems responsible for oxygen uptake. At no time during this period will addition of TCA cycle intermediates (with the exception of succinate as discussed above) alter the Q_{O_2}. If oxygen uptake is taking place in the TCA cycle, it follows that a continuous supply of dicarboxylic acids is available, either through CO_2 fixation or by transamination reactions involving alpha ketoglutarate. The intracellular store of carbohydrate which is being oxidized must be of considerable size. Glycogen is histologically demonstrable in the tissue and, chemically, is present in adequate amount to maintain the observed Q_{O_2} for over 8 hours. The content is some 4.5 mg per gm wet weight, equivalent to 25 moles of glucose and requiring 150 moles of oxygen for complete oxidation. Based on a Q_{O_2} of 18.4 moles/gm wet wt/hr., it would therefore be adequate for just over 8 hours if the lactate initially formed were later metabolized.

The glycogen content was determined before and after a 2-hour incubation and the results are given in Table V, expressed as the quantity of glycogen hydrolyzed, i.e. no longer precipitable with alcohol, with its calculated glucose equivalent. By expressing the lactic acid production and the Q_{O_2} in the same manner, a striking

TABLE V. ENDOGENOUS AND EXOGENOUS SUBSTRATE UTILIZATION
(Phosphate medium; 100 per cent O_2; mean values of 6 to 12 flasks)

	μmoles/gm/2 hr.	Glucose equivalent (μmoles/gm/2 hr.)
Endogenous		
Glycogen hydrolysis	2.30[a]	12.8
Lactic acid production	13.8	6.9 } 12.4
Q_{O_2}	3.3	5.5 }
Exogenous[b]		
Glucose uptake	13.0	13.0
Lactic acid production	26.1	13.1

[a] Mg/gm wet weight.
[b] Glucose, 30 mg %.

equivalence between the two is obtained. Though this is not conclusive proof, it is suggestive evidence that glycogen is indeed the principal, and possibly the only, endogenous substrate present.

Attempts were made to deplete the endogenous glycogen to permit a study of the utilization patterns of exogenous substrates. However, aeration for a period of up to 4 hours, with medium changes to eliminate the lactate formed, did not accomplish this. Longer periods of aeration may well be adequate, but deterioration of the tissue under such circumstances raises questions concerning the validity of the data so obtained.

Exogenous glucose uptake is also related to glycolysis (Table V). Uptake was determined by difference after a 2-hour incubation, and the fact that an equivalent amount of lactic acid is produced indicates that exogenous glucose is metabolized entirely to lactic acid.

Iodoacetate, at a concentration of 2×10^{-3} M (sufficiently high to inhibit completely lactic acid production), reduces the Q_{O_2} to zero. This would suggest, as a first approximation, that oxygen uptake is completely supported by the utilization of a carbohydrate substrate. Conclusions from data obtained by the use of iodoacetate must be interpreted with caution, however. In any event, no conclusion may be drawn as to the sequences involved. Iodoacetate may well restrict carbohydrate oxidation in the hexose monophosphate shunt shown to be present in bone (Cohn and Forscher, 1962*a*, 1962*b*) if the cell is dependent for reoxidation of the reduced TPN formed in the first two stages upon coupling the reoxidation with either the DPN formed in the pyruvate-to-lactate step or the TPN formed in the malic enzyme reaction. Ability of the cell to utilize the TPN formed in synthetic mechanisms or transamination reactions would, of course, relieve it of dependence on later glycolytic intermediates as electron acceptors.

Uncoupling

Uncoupling of oxidative phosphorylation in a tissue can be accomplished with 2,4-dinitrophenol (DNP) at a concentration of 2×10^{-4} M. With DNP, there was an increase in the Q_{O_2} of some 50 per cent and an increased accumulation of lactic acid under aerobic conditions (Table VI). Both endogenous and exogenous lactate pro-

TABLE VI. The Effects of an Uncoupler on Glycolysis
(Glucose 17 mM; 2,4-DNP 2×10^{-4} M; bicarbonate medium)

Additions	Lactic acid production (μmoles/gm/hr.)	
	Aerobic	Anaerobic
None	14.2	39.1
Dinitrophenol	37.1	36.1

duction were increased to that found under anaerobic conditions. There was a slight increase in the quantity of exogenous glucose taken up by the tissue, but not enough to account for the increased lactic acid production. It would seem that, in the presence of dinitrophenol, the endogenous supply of carbohydrate is more rapidly glycolyzed. These data also support the conclusion that the TCA cycle is functioning in this tissue and that it is, as in all tissues studied with this uncoupler, intimately linked with glycolysis as far as control is concerned. This point is also deducible from the fact that the Pasteur effect is present both endogenously and when external substrate is added.

Parathyroid Action

The parathyroid hormone, a purified preparation kindly donated by Dr. Rasmussen, was added *in vitro* at a level of 5 units per ml, and the various parameters so far described were studied. Because of the increase in citrate production observed *in vivo* (Firschein *et al.*, 1958), it was surprising that no significant alteration in citric acid levels was observed, either in the medium or in the tissue. No effect was noted on glucose uptake or on the Q_{O_2} in phosphate medium. No significant changes in calcium or phosphate levels in the medium could be demonstrated.

The only significant finding was that shown in Table VII, an increase in the accumulation of lactate in the system. This was found to occur in phosphate and in bicarbonate medium, at low and high levels of glucose, but only under aerobic conditions. The differences between the means, in phosphate medium, have p values of about 0.05. If comparisons are made of paired flasks containing the alternating halves of the same calvaria, the p values are less than 0.02.

TABLE VII. The Effects of Parathyroid Hormone on Lactate Production

Conditions			Q_L[a] (μmoles/gm/hr.)		
Medium	Gas[b]	Glucose (mM)	Control	PTH (5 u/ml)	Increase (%)
Phosphate	Air	1.6	12.7 ± 0.6 (4)	15.0 ± 0.4 (4)	18
Phosphate	Air	17.0	18.9 ± 0.5 (4)	21.1 ± 0.3 (4)	12
Bicarbonate	N_2	17.0	35.0 ± 0.4 (8)	34.5 ± 0.8 (8)	0
Bicarbonate	O_2	0	5.2 ± 0.2 (4)	6.3 ± 0.3 (4)	21
Bicarbonate	O_2	1.6	13.5 ± 0.5 (4)	17.0 ± 0.5 (4)	25
Bicarbonate	O_2	13.0	16.1 ± 0.4 (4)	19.4 ± 0.5 (4)	21
Bicarbonate	O_2	17.0	15.2 ± 0.3 (12)	18.9 ± 0.4 (12)	24[c]

[a] Means ± standard error; numbers of flasks in parentheses.
[b] N_2 and O_2 contained 5 per cent CO_2 for buffering.
[c] Significant at $p < 0.001$.

In bicarbonate medium, the findings are obviously significant. The absence or presence of glucose and the level at which it is present seem to be of no detectable importance.

On the hypothesis that the hormonal effect could well be time dependent, longer incubation periods have been tried (Fig. 3A). A continuing effect was observed, not increasing but rather decreasing with time. Similarly, with variation in concentration of the hormone, a dose response curve is obtained (Fig. 3B). The response can no longer be produced if the hormone is allowed to undergo inactivation by being removed from the deep freeze for a few weeks.

Conclusion

Calvarium in these studies behaved qualitatively in a fashion similar to metaphyseal chips: low oxygen consumption, excess reserve succinate dehydrogenation capacity, high glycolytic capacity, and,

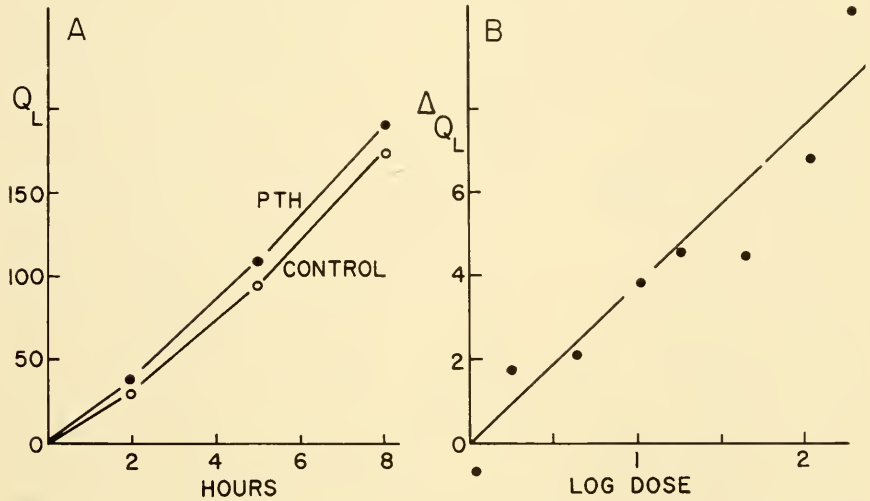

FIG. 3. The effects of parathyroid hormone on lactate production by calvaria. A, Q_L, μmoles per gm wet weight over 8 hours in the presence (5 units per ml) and absence of parathyroid hormone. B, the relation between parathyroid hormone concentration and lactate production; Δ_{Q_L} represents the difference, in μmoles gm hr., between paired control incubations and the indicated dosage of parathyroid hormone expressed as log units per ml + 1. Thus, the highest dose corresponded to 45 units per ml of Rasmussen polypeptide.

despite a Pasteur effect, a high rate of *aerobic* glycolysis (Laskin and Engel, 1956; Borle *et al.*, 1960*a*; Cohn and Forscher, 1961). Though these studies represent the first reported (Neuman and Dowse, 1961) metabolic effects in bone resulting from purified parathyroid hormone added *in vitro*, the effects were similar to those observed in metaphyses from animals given massive doses of hormone parenterally (Laskin and Engel, 1956; Borle *et al.*, 1960*a*; Cohn and Forscher, 1961).

In view of the rather profound physiological effects of parathyroidectomy in most species, the observed metabolic changes from excess hormone seem rather minor. In fact, the number of parameters which are *unaffected* is remarkable and suggestive in itself: no change in Q_{O_2}, no change in glucose uptake, no change in citrate production, no change in lactate production anaerobically.

One gains the impression that any hormonal effects on carbohy-

drate metabolism are *indirect* and, in large measure, dependent upon experimental conditions. Thus, in intact dogs, citrate production by bone is increased (Firschein *et al.*, 1958); in calvarium and metaphyseal chips no effects are seen *in vitro* sometimes (Borle *et al.*, 1960*a*; Neuman and Dowse, 1961; Cohn and Forscher, 1961), though significant increases in labeled citrate (from labeled pyruvate, Lekan *et al.*, 1960) or labeled CO_2 (from C-6–labeled glucose via the TCA cycle, Cohn and Forscher, 1962*b*) are also reported.

The most consistent finding is increased aerobic production of lactate, and this, too, is not a dramatic change, usually 20 to 30 per cent (Laskin and Engel, 1956; Borle *et al.*, 1960*a*; Neuman and Dowse, 1961). Such a small shift in glycolysis has a number of plausible metabolic explanations, among which increased intracellular levels of inorganic phosphate (P_i) is perhaps most appealing (Egawa and Neuman, 1963). Surely P_i can be a controlling factor between competing glycolytic and oxidative mechanisms. Increased P_i could increase aerobic lactate production and could also explain variable condition-dependent changes noted above in the operation of the TCA cycle.

If we assume, for the moment, that parathyroid hormone does increase P_i, this change is in itself likely to be the result of some more primary event such as a change in transport mechanisms of ions in and out of the cell.

This kind of speculation led us to examine the movement of labeled inorganic phosphate in a variety of tissues, particularly bone, kidney, and intestine. Hope for some modest success rested in the classic phosphaturic activity ascribed to the hormone (Albright and Reifenstein, 1948). Indeed, it has been possible to show significant alterations in the movement of labeled phosphate in all three tissues (Egawa and Neuman, 1963; Borle *et al.*, 1963). Of particular interest, here, are studies on calvarium. Because calvarium contains a fair quantity of bone mineral which represents a large pool of exchangeable phosphate, it was not possible to sample the cellular inorganic phosphate directly. Instead, the acid-soluble ester fraction was isolated as an indicator of intracellular label. As shown in Fig. 4, there was a significant increase in labeling of the ester-P fraction in response to the hormone.

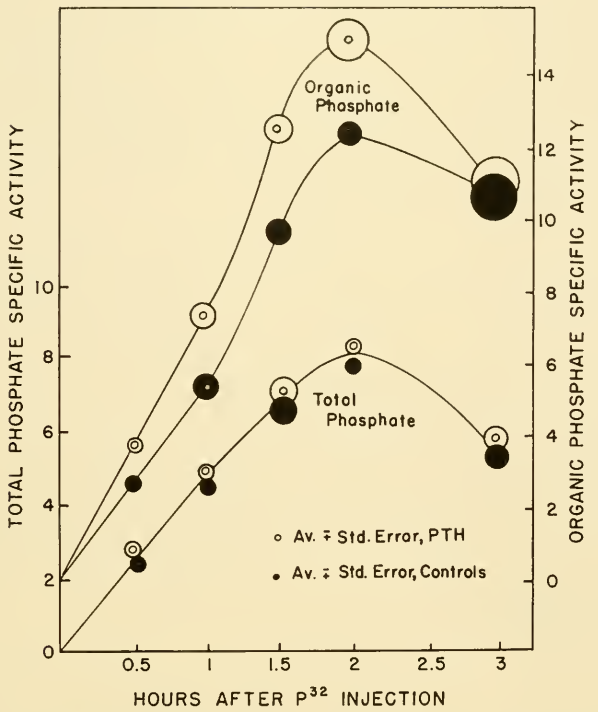

FIG. 4. The increased incorporation of labeled phosphate into the acid-soluble esters of calvarium after parathyroid hormone. Newborn rat pups were given 10 units Lilly Para-Thor-Mone each, or control injections, 30 minutes prior to labeled phosphate. The size of the circles indicates the standard error for each point. (Data taken from Egawa and Neuman, 1963.)

Thus, it is our present opinion that explorations of carbohydrate metabolism have not yet yielded important clues as to the primary sites of action of parathyroid hormone. Whether further exploration of phosphate transport will prove more profitable, only time will tell.

SUMMARY

Calvaria from newborn rats were taken for biochemical study of the action of parathyroid hormone for several reasons: responsiveness of the tissue, its low mineral–high cell content, its low marrow contamination, and its ease of preparation with little cell damage.

Though control studies indicated the operation of the Krebs cycle as the principal oxidative pathway, the most striking metabolic properties of calvaria were: almost exclusive utilization of glucose (or glycogen), a high and medium-dependent glycolytic rate, a high aerobic glycolysis (incomplete Pasteur mechanism), and a low but medium-independent oxidative rate.

Purified parathyroid polypeptide added to the medium showed relatively little influence on metabolic activity. There were no significant changes in citrate production or utilization, glucose uptake, Q_{O_2}, or levels of Ca^{++} or inorganic phosphate in the medium. The only significant finding was an increased production of lactate, and this only under aerobic conditions.

The impression is gained that the action of the hormone on this tissue under conditions *in vitro* may be on some system other than glucose metabolism, and that the metabolic changes, though definite, are small and indirect in character. Arguments are presented, along with preliminary evidence, that the hormone may influence the transport of phosphate in and/or out of the cell.

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Some Chemical Factors Influencing Bone Resorption in Tissue Culture

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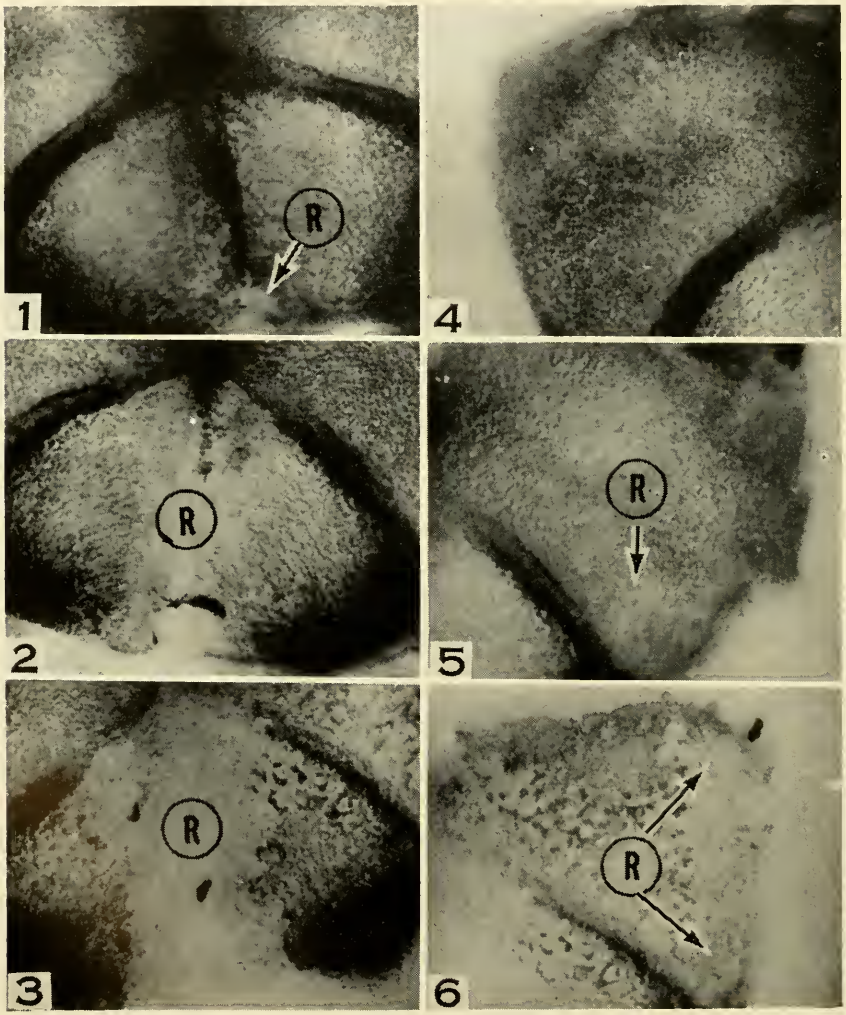
DESPITE the pioneering work of Gaillard (1955*a*, 1955*b*, 1957, 1961*a*, 1961*b*), who demonstrated a direct effect of parathyroid tissue or extract on bone in tissue culture, others, including ourselves, were not able to obtain similar evidence until most recently. In the course of our work, however, we did find a method of producing bone resorption routinely in tissue culture, *without* adding parathyroid extract to our system, thereby permitting investigation of some of the morphological and biochemical changes related to bone resorption per se. Of particular interest to us was the development of a tissue culture system which would enable us to study the mechanism of action of potential stimulators or inhibitors of resorption. The purpose of the present paper is to describe the development of such a system and some of the significant chemical factors which have been found to exert a pronounced effect on the phenomenon of bone resorption.

Observations

Regulation of Bone Resorption by Oxygen Tension

In our earlier experiments (Goldhaber, 1958, 1960) it was found that bone resorption could be induced within young mouse calvaria

grown in stationary Leighton tubes by introducing high concentrations of oxygen into the gas phase every 2 to 3 days at the time of refeeding the cultures. By using various oxygen concentrations in the gas mixture (from 0 to 100 per cent) it has been possible to demonstrate a direct correlation between oxygen concentration and extent of bone resorption (Goldhaber, 1961). Furthermore, on



FIGURES 1 TO 6

changing to roller tubes it was found that good bone resorption took place with hitherto ineffective concentrations of oxygen, whereas high concentrations of oxygen were toxic. Of interest was the finding that variation in extent of bone resorption (from slight to marked) could be obtained in roller tubes by utilizing a relatively small spectrum of oxygen concentrations. From Figs. 1 to 6 it may be seen that after 7 days of culture, whereas gassing with 10 per cent oxygen resulted in only slight resorption in the median suture area of the frontal bone, 20 per cent oxygen resulted in almost complete resorption of this region. In both instances, however, the parietal bone was relatively unaffected. On the other hand, cultures gassed with 30 per cent O_2 revealed widespread resorption in the median suture area, occasionally affecting the parietal bones as well. It seems clear, therefore, that in this culture system bone resorption not only is oxygen dependent but can be *regulated* by alterations in the oxygen concentration.

Observations of Bone Resorption with Time-Lapse Microcinematography

Attempts to visualize the resorptive process with time-lapse microcinematography at magnifications up to 660 times have revealed

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FIG. 1. Seven-day roller-tube culture gassed with 10 per cent oxygen (balance nitrogen). Note slight resorption (*R*) in sagittal suture of frontal bone area. (× 14.)

FIG. 2. Seven-day roller-tube culture gassed with 20 per cent oxygen (balance nitrogen). Note moderate amount of resorption (*R*) in sagittal suture area of frontal bone. (× 14.)

FIG. 3. Seven-day roller-tube culture gassed with 30 per cent oxygen (balance nitrogen). Note marked resorption (*R*) in sagittal suture area of frontal bone. (× 14.)

FIG. 4. Parietal bone of same calvarium as Fig. 1. Culture gassed with 10 per cent oxygen (balance nitrogen). Note absence of resorption. (× 14.)

FIG. 5. Parietal bone of same calvarium as Fig. 2. Culture gassed with 20 per cent oxygen (balance nitrogen). Slight resorption (*R*) evident. (× 14.)

FIG. 6. Parietal bone of same calvarium as Fig. 3. Culture gassed with 30 per cent oxygen (balance nitrogen). Note marked resorption (*R*). (× 14.)

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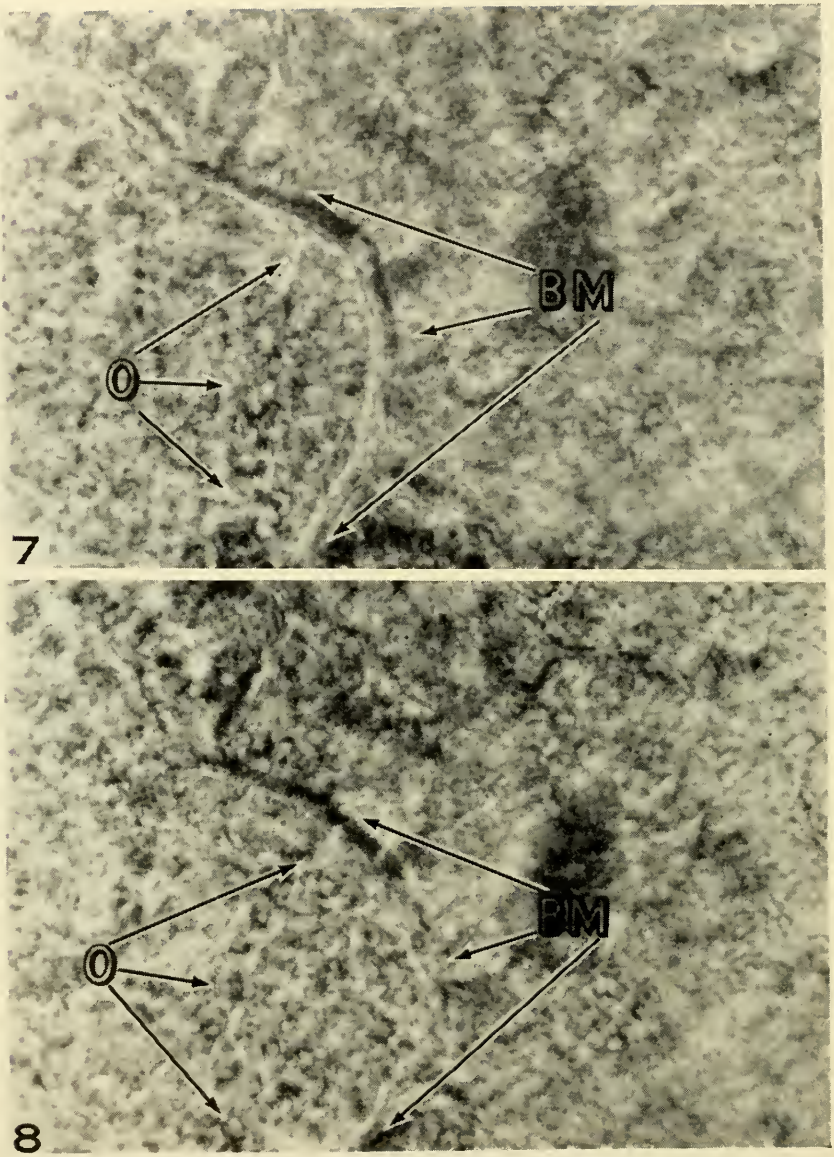

FIGURES 7 AND 8

that both macrophages and osteoclasts are capable of resorbing bone (Figs. 7 and 8). The formation of Howship's lacunae, however, is a task restricted to the osteoclasts. It is of interest that large numbers of macrophages appear relatively late in the culture period and that, to date, we have not observed any fusion of these mononucleated cells to form large, multinucleated osteoclasts. Although we have observed osteoclastic activity as early as 1 day in culture, it is probable that these cells were present at the time of explantation and were stimulated by the experimental conditions rather than being formed *de novo*. With regard to the fate of the osteoclast, the longest period that we have been able to follow one cell has been approximately 48 hours, during which time the cell appeared to enlarge and become more granular. In several instances we have observed the death of such granular osteoclasts, which was manifested by a sudden gelation of the cytoplasm and cessation of cell movement. Significantly, further bone resorption did not continue at these sites. The aggressive action of these cells against the resorbing bone surface supports the idea that osteoclasts actively participate in the process of bone resorption.

The hypothesis that osteoclasts actively secrete some product involved in bone resorption is further supported by some of our time-lapse studies, wherein it is possible to visualize the production of large vacuoles at the border of the resorbing bone. Occasionally, in cultures exhibiting extremely rapid and marked bone resorption, the presence, growth, and fusion of huge bubblelike vacuoles has been noted in resorbing areas at the height of resorptive activity. Time-lapse films of such giant "bubbles" suggest that these unusual structures are intracytoplasmic. Their sudden, rapid contraction several

FIG. 7. Four-day living culture, showing osteoclast (*O*), outlined by arrows, in Howship's lacunae. *BM*, bone margin. (Enlargement of 16-mm frame, × approx. 540.)

FIG. 8. Same field, 6½ hours later, showing resorption of some of the bone margin (*BM*) as the osteoclast (*O*) burrows deeper into the bone. (Enlargement of 16-mm frame, × approx. 540.)

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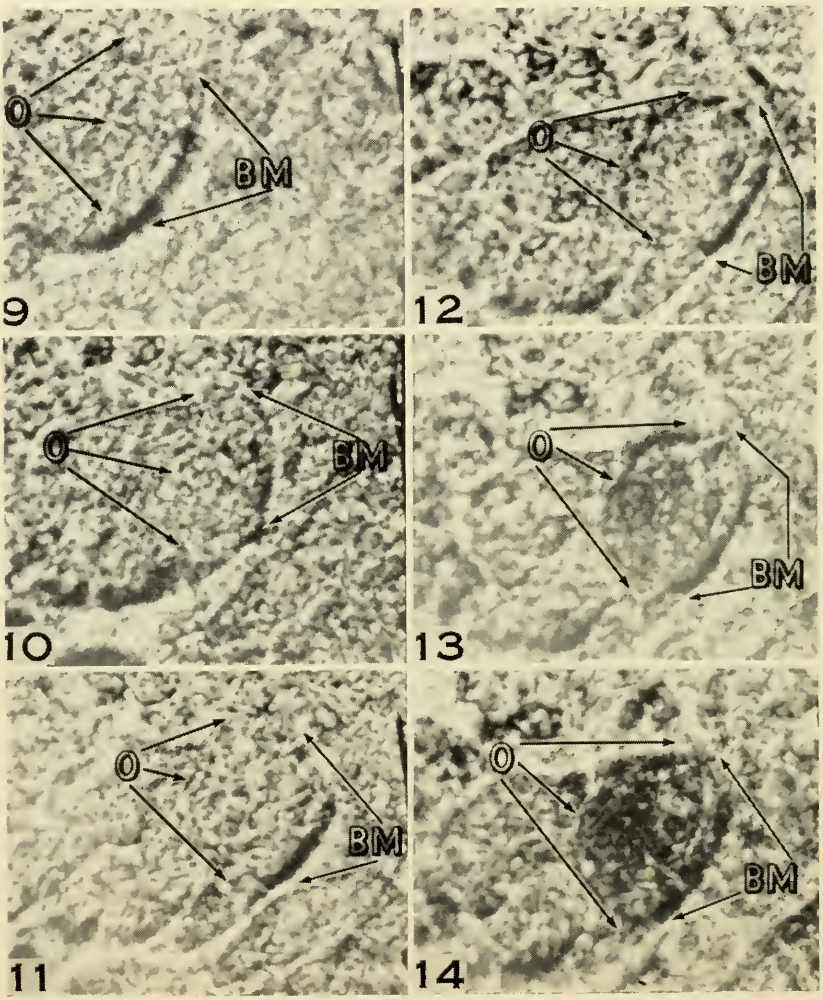
hours after reaching maximum size is reminiscent of the contractile vacuole of unicellular organisms.

The presence of large amounts of succinic dehydrogenase demonstrated histochemically in osteoclasts has been reported independently by several laboratories simultaneously (Schajowicz and Cabrini, 1960; Burstone, 1960; Goldhaber and Barrnett, 1960). The



FIGURES 9 TO 14

study by Goldhaber and Barrnett (1960), however, was carried out on resorbing bone tissue cultures under time-lapse microcinematography. This procedure permitted visualization of active osteoclastic bone resorption prior to the histochemical staining of the very same osteoclasts (Figs. 9 to 14). In view of the intimate association of this enzyme with the mitochondria, the likelihood appears that osteoclasts have a high metabolic activity, supporting the interpretation of previous cinematographic evidence concerning the active role of osteoclasts in the process of bone resorption.

Some Chemical Inhibitors of Resorption

The high energy requirement of bone-resorbing cells is suggested by the finding that addition to the culture medium of 2,4-dinitrophenol (5×10^{-5} M) inhibits resorption during the 2-week culture period, presumably by inhibiting synthesis of adenosine triphosphate (Figs. 15 and 16). Under these conditions, bone *formation* was not totally inhibited (Figs. 17 and 18). Of further interest was the finding that addition to the medium of sodium malonate (5×10^{-3} M), a powerful inhibitor of succinic dehydrogenase, inhibits bone resorp-

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FIG. 9. Two-day resorbing culture showing indistinct osteoclast (*O*) against hollowed-out bone margin (*BM*). (Portion from enlargement of 16-mm frame, × approx. 400.)

FIG. 10. Continuation from Fig. 9. Active resorption proceeding, as may be seen from new position of osteoclast (*O*) and the resorbing bone margin (*BM*). (× approx. 400.)

FIG. 11. Continuation from Fig. 10. Resorption still progressing. Note new position of osteoclast (*O*) and adjacent bone margin (*BM*). Total time interval between Fig. 9 and Fig. 11 was 16½ hours. (× approx. 400.)

FIG. 12. Continuation from Fig. 11 after reaction mixture for histochemical disclosure of succinic dehydrogenase was added to tissue culture medium. Note that outline of osteoclast (*O*) is becoming more distinct as a result of the initiation of the color reaction. *BM*, bone margin. (× approx. 400.)

FIG. 13. Continuation from Fig. 12. Development of color reaction proceeding in osteoclast (*O*). No further bone resorption occurring owing to toxicity of the histochemical procedure. *BM*, bone margin. (× approx. 400.)

FIG. 14. Continuation of histochemical reaction for succinic dehydrogenase. Note intensity of color reaction in osteoclast (*O*). Time interval between Fig. 12 and Fig. 14 was 8 hours. *BM*, bone margin. (× approx. 400.)

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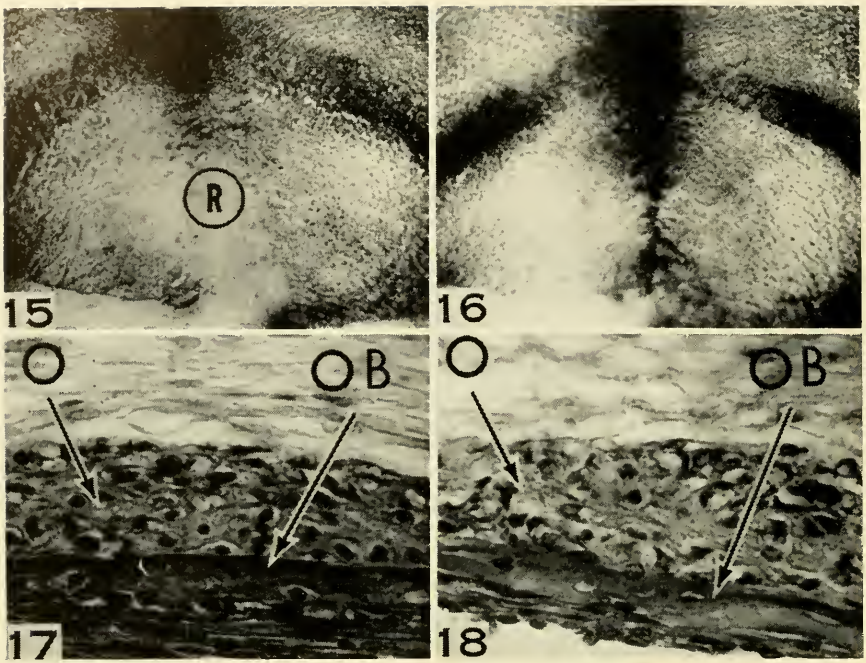


FIG. 15. Six-day control calvarium (roller tube at 20 per cent O_2). Note resorption (*R*) in median suture area. (× 14.)

FIG. 16. Six-day culture containing approximately 5×10^{-5} M 2,4-dinitrophenol (roller tube at 20 per cent O_2). Note inhibition of resorption, although outgrowth is healthy. (× 14.)

FIG. 17. Histological section through control calvarium maintained in culture for 2 weeks. Note thick layer of new osteoid (*O*) on surface of darker, original bone (*OB*). (× 325.)

FIG. 18. Histological section through 2-week culture exposed continuously to 5×10^{-5} M 2,4-dinitrophenol. Note patch of defective osteoid (*O*) on surface of original bone (*OB*). (× 325.)

tion (Figs. 19 and 20). Again, under these conditions, bone formation was not completely inhibited (Figs. 21 and 22), suggesting that bone formation and resorption are independent processes having different metabolic pathways. The above findings support the concept that the Krebs cycle is intimately involved in the process of bone resorption. Along these lines, previous concomitant chemical analyses of the supernatant fluid of resorbing bone cultures have revealed a cumulative increase in citric acid over a 10-day culture

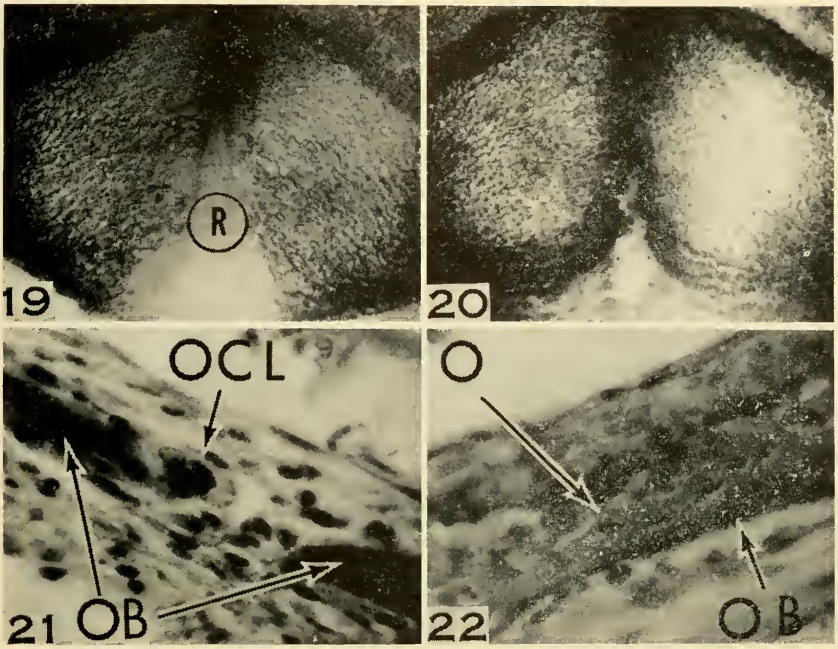


FIG. 19. Seven-day control calvarium (stationary tubes gassed with 95 per cent $O_2 + 5$ per cent CO_2). Note resorption (*R*) in median suture area. (× 14.)

FIG. 20. Seven-day culture containing 5×10^{-3} M sodium malonate (stationary tubes gassed with 95 per cent $O_2 + 5$ per cent CO_2). Note inhibition of resorption. (× 14.)

FIG. 21. Histological section through control calvarium maintained in culture for 9 days. Note multinucleated osteoclast (OCL) at edge of original bone spicule (*OB*). (× 325.)

FIG. 22. Histological section through 9-day culture exposed continuously to 5×10^{-3} M sodium malonate. Note layer of new osteoid (*O*) on surface of original bone (*OB*). (× 325.)

period, amounting to 8 to 19 times the total citric acid content of the original calvaria (Kenny *et al.*, 1959).

Effect of Parathyroid Extract

Recently, we have found that it is possible to *partially* inhibit the resorption of bone in response to oxygen by eliminating the embryo extract component of the supernatant. Surprisingly, with this new culture medium (80 per cent horse serum and 20 per cent Gey's

balanced salt solution containing penicillin and streptomycin) we have been able to demonstrate a dramatic, clear-cut effect of Lilly parathyroid extract in roller-tube cultures gassed with 50 per cent O_2. This finding differed from our previous results in that resorption did not cease after approximately 5 days, but continued until practically all the bone was destroyed. The gross, massive bone resorption resulting from the addition of 0.5 unit of parathyroid extract per ml of medium every 2 days for 15 days may be seen in Fig. 23.

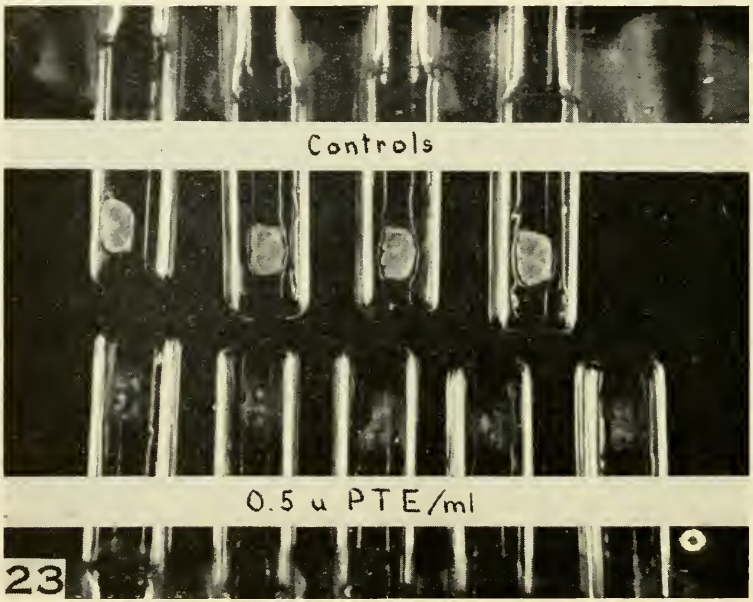


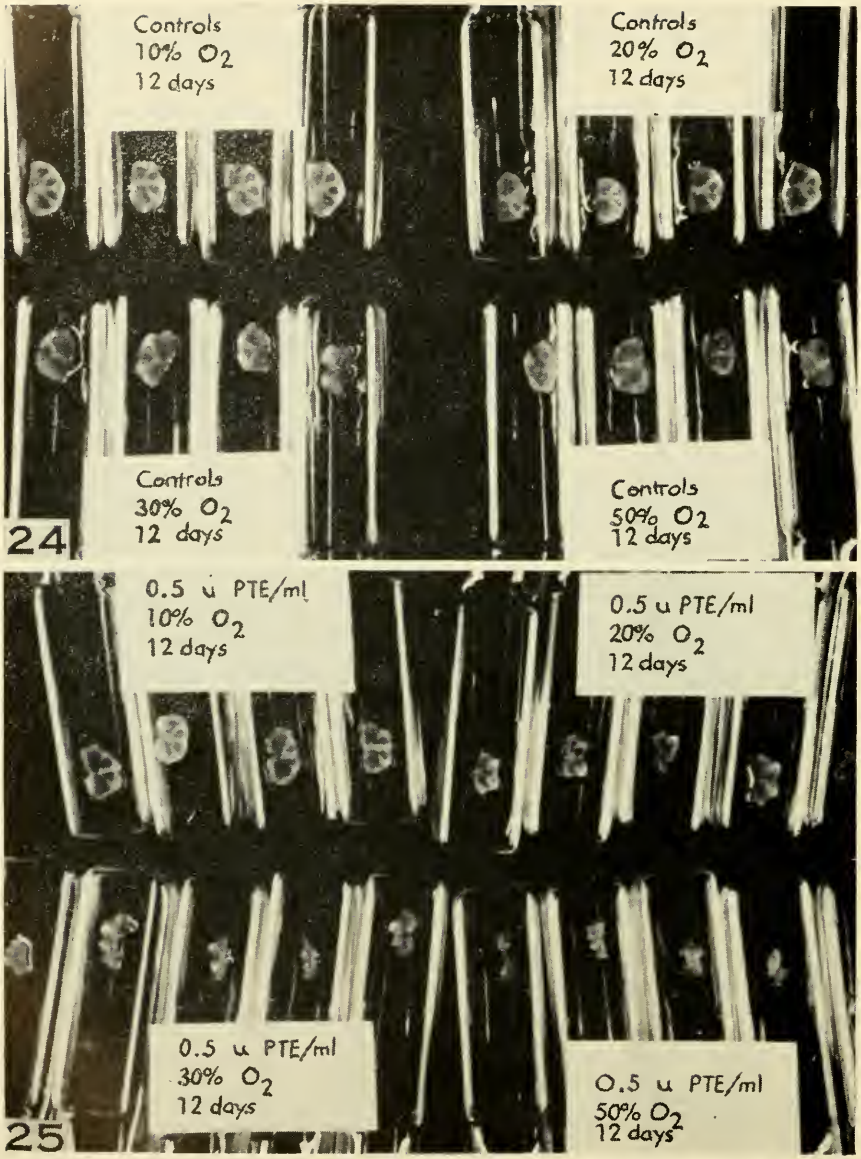
FIG. 23. Gross appearance of 15-day cultures. Both control and experimental roller tubes were gassed with 50 per cent O_2. Note almost complete resorption of calvaria in tubes containing 0.5 unit of Lilly parathyroid extract per ml of medium (80 per cent horse serum + 20 per cent Gey's balanced salt solution).

It should be noted that both the control and experimental groups were gassed with 50 per cent O_2. Evidence that this effect was due to the hormone per se rather than to some contaminant in the commercial parathyroid extract was obtained by the use of more highly purified parathyroid preparations* which gave similar results.

* Kindly provided by Dr. Paul L. Munson.

In view of our earlier results with chick embryo extract–containing medium, wherein it was possible to demonstrate regulation of extent of bone resorption by alteration of oxygen tension, it was significant that the response to parathyroid extract could be altered by variation in oxygen tension. From Figs. 24 and 25 it may be seen that whereas increasing the oxygen tension from 10 per cent to 50 per cent had little effect on the gross appearance of 12-day control cultures of mouse calvaria, there was a distinct effect on the experimental cultures containing 0.5 unit of parathyroid extract per ml of medium. Although the hormone-containing group gassed with 10 per cent oxygen showed little difference from its corresponding control group without hormone, the experimental groups gassed with 20 per cent, 30 per cent, and 50 per cent O_2 showed graded enhancement of bone resorption in response to increasing oxygen tensions.

The *interdependence* of oxygen tension and parathyroid extract in stimulating bone resorption in this system was demonstrated by the observation that despite optimum oxygen tensions (50 per cent O_2) in the gas phase, decreased bone resorption resulted from decreasing the concentration of parathyroid extract in the medium (Fig. 26). Although cultures gassed with 50 per cent O_2 and exposed to 0.01 or 0.05 unit PTE per ml showed no gross difference from control tubes not receiving hormone, those treated with 0.1 or 0.5 unit PTE per ml (and gassed with 50 per cent O_2) showed enhanced bone resorption after 2 weeks in culture. It should be noted that distinct differences in extent of resorption for the different dose levels of parathyroid extract were determined readily during the 1st week of culture by microscopic examination and scoring of the living cultures. Another means of comparing the effects of different doses of parathyroid hormone on bone resorption in the previous experiment was to perform calcium determinations (Munson *et al.*, 1955) on all supernatants throughout the experiment. From Fig. 27 it may be seen that the largest differences in calcium level occurred in the media collected at the 4th, 6th, and 8th days of culture. This interval corresponded to the period of most rapid bone resorption. Although no statistical analysis was performed, it appears that during this rapid resorption period the calcium levels in the media of tubes containing 0.5, 0.1, and 0.05 unit of PTE per ml were significantly



FIGURES 24 AND 25

different from one another and from the control tubes without hormone. In view of its sensitivity to small quantities of parathyroid hormone, the present tissue culture system may provide a simple assay method for the hormone, based upon scoring severity of resorption microscopically. This approach has been of practical value already in studies concerning the extraction of parathyroid hormone from rat and human parathyroids.*

Citrate and Lactate Production in Resorbing and Nonresorbing Bone Cultures

In a previous study (Kenny *et al.*, 1959) it was shown that the media from our resorbing bone cultures showed consistent increases in citric acid, calcium, and phosphorus, which paralleled the bone resorption seen morphologically. Lactic acid, on the other hand, accumulated in the nonresorbing, control cultures. Whether the accumulation of citric acid in the media of the resorbing cultures and lactic acid in the media of the nonresorbing cultures was significant with respect to the mechanism of bone resorption was not known. This pattern of acid accumulation might have been coincidental, for in those studies bone resorption was initiated by gassing the experimental stationary cultures with 95 per cent O_2 and 5 per cent CO_2, whereas the nonresorbing stationary cultures were maintained by gassing with 95 per cent air and 5 per cent CO_2. It was to be expected, therefore, that the experimental cultures would favor aerobic metabolism and citrate production, whereas the control cultures would favor anaerobic metabolism and lactic acid formation.

The striking ability of parathyroid extract to stimulate bone resorption in our tissue culture system (both control and experimental

* Studies in progress in collaboration with Drs. Paul L. Munson, Philip F. Hirsch, and Armen J. Tashjian.

FIG. 24. Gross appearance of control roller tubes (12 days in culture) gassed with 10, 20, 30, or 50 per cent oxygen. It should be noted that some resorption was evident *microscopically* in the tubes gassed with the higher concentrations of oxygen.

FIG. 25. Gross appearance of experimental roller tubes (12 days in culture) containing 0.5 unit of parathyroid extract per ml of medium. Note the increasing effectiveness of the hormone on resorption as the oxygen tension is increased.

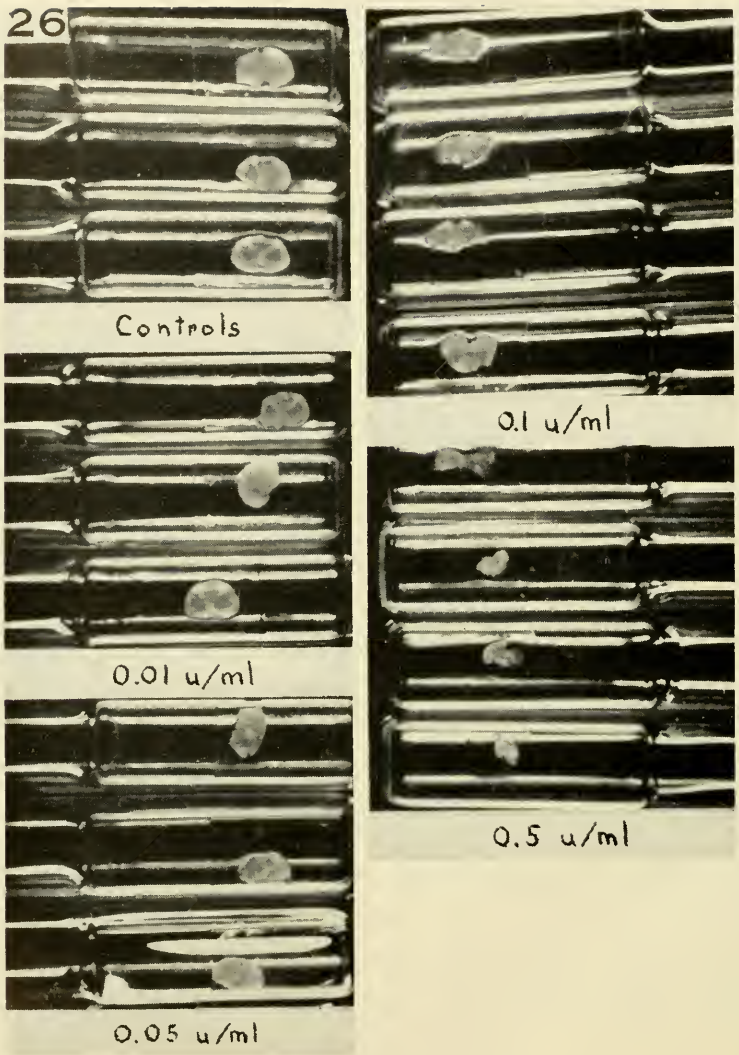


FIG. 26. Appearance of 2-week roller-tube cultures treated with various concentrations of parathyroid extract and gassed with 50 per cent O_2. Gross effects may be noted at the level of 0.1 u/ml and 0.5 u/ml. *Microscopically*, 0.05 u/ml of hormone caused some enhancement of resorption.

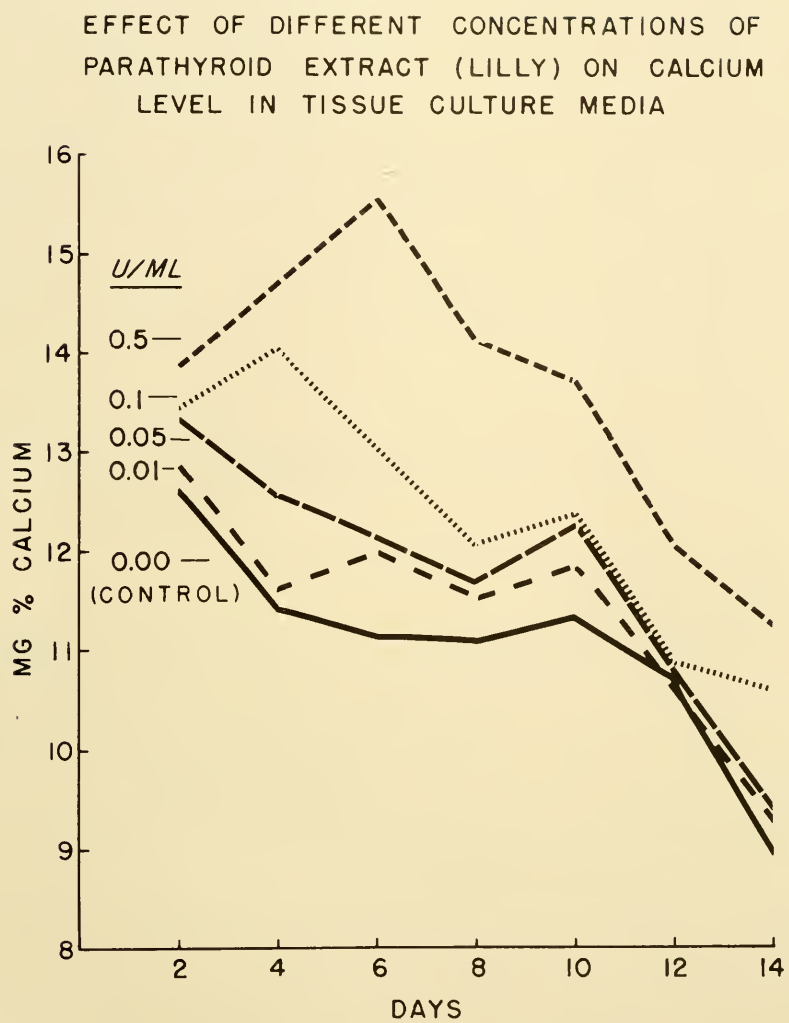


FIG. 27. Graph showing calcium levels in media removed from same experiment as shown in Fig. 26. Note that the increased calcium levels obtained with graded doses of parathyroid extract reflect the bone resorption observed morphologically.

tubes being gassed with 50 per cent O_2) led us to reinvestigate the question of citrate versus lactate production by resorbing calvaria. This work was done in collaboration with Dr. George R. Martin's laboratory and will be published elsewhere (Mecca *et al.*, 1963).

Briefly, it was found that citrate levels were greatly increased in the media from parathyroid-treated cultures and closely paralleled the rise in calcium and phosphate levels found throughout the experiment. On the other hand, lactate levels were raised in the hormone-treated cultures only during the first 4 days of the experiment. By the 6th day, there was essentially no difference between experimental and control cultures. During the remainder of the experiment the nonresorbing, control calvaria produced more lactate than the resorbing, hormone-treated group (Fig. 28A). Apparently, lactate production by both the experimental and control cultures reflected glucose utilization.

It appears, therefore, that of the two acids, citrate probably plays a greater role in resorption than lactate—a conclusion reached by Kenny *et al.* (1959) utilizing a tissue culture system wherein exogenous parathyroid extract was not required for resorption.

Bone Collagen Degradation Products from Resorbing Calvaria

Attempts to identify and characterize bone collagen degradation products produced in tissue culture as the result of bone resorption are being made in collaboration with Dr. Melvin J. Glimcher's laboratory. Full details will be presented elsewhere (Stern *et al.*, 1963*a*, 1963*b*). Briefly, young mouse calvaria were labeled *in utero* with either proline-C^{14} or proline-H^3. Six days after birth the calvaria of the litters were removed and set up in roller-tube cultures with and without parathyroid extract. All tubes were gassed with 50 per cent O_2. The cultures were examined daily and scored for resorption. The supernatant fluid was collected separately from each culture tube every 2 days for chemical analysis. The morphological changes developed as expected: during the 2-week culture period the controls showed minimal resorption, whereas the parathyroid-treated cultures were almost totally resorbed.

Following 6 N HCl, 105°C hydrolysis for 24 hours, and chromatographic separation of proline and hydroxyproline from the pooled media of each 2-day period, it was found that there was more labeled hydroxyproline in the media from the parathyroid-treated samples than in the nonresorbing controls (Figs. 28B, 28C). The peak ac-

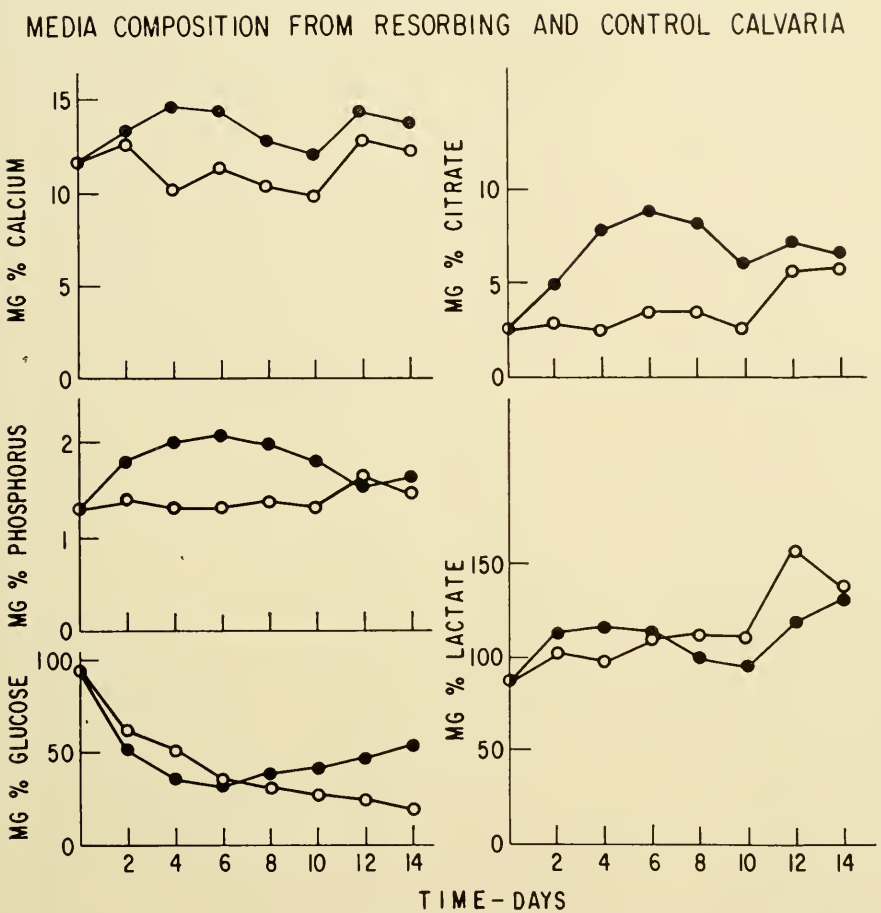


FIG. 28A. Comparison of media of parathyroid extract (0.5 u/ml) resorbing cultures versus those of control, relatively nonresorbing cultures with respect to calcium, phosphorus, citrate, glucose, and lactate over a 2-week culture period. Note that the calcium and citrate levels of the PTE resorbing cultures (solid circles) are consistently elevated above the controls (open circles), and that the lactate levels of the controls are higher than those of the parathyroid extract–treated cultures after 6 days. (Data reproduced from Mecca *et al.*, 1963.)

tivity occurred in the media collected on the 4th day, which corresponds to the period of most rapid resorption morphologically. Fractionation of whole media without prior hydrolysis showed that the labeled hydroxyproline was not in a large peptide or protein

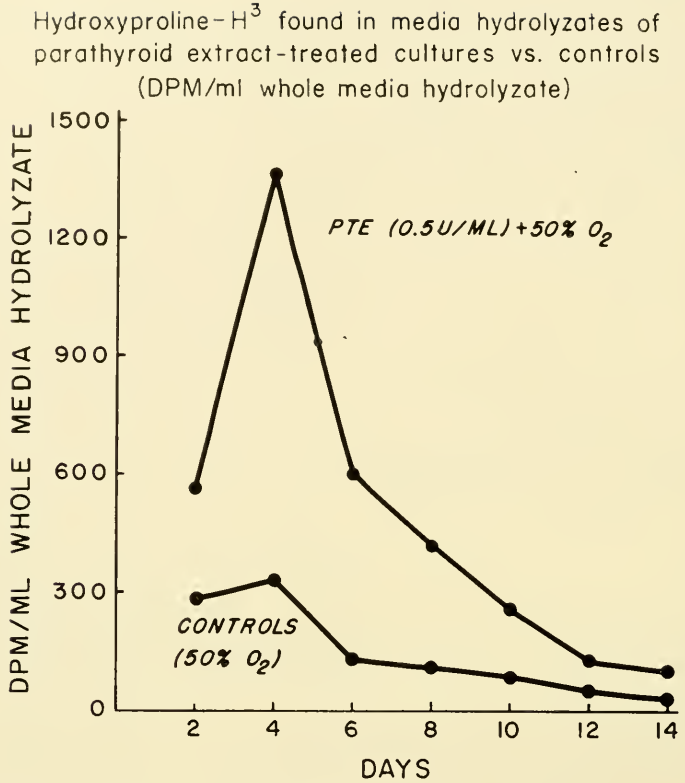


FIG. 28B. Graph of hydroxyproline-H^3 (DPM/ml whole media hydrolyzate) found in media of parathyroid extract–treated cultures versus controls. Note that peak release of labeled collagen occurs in media collected on the 4th day, which corresponds roughly to the period of most rapid bone resorption, as visualized morphologically or as measured by determining the calcium level in the media. (Data reproduced from Stern *et al.*, 1963*a*, 1963*b*.)

form, but was recoverable both as small peptides and as the free amino acid.

Effect of Vitamin A

It is well known that hypervitaminosis A can intensify bone resorption *in vivo*, leading to bone deformities and fractures in the rat (Barnicot and Datta, 1956). Although Barnicot's experiment concerning the intracerebral implantation of combined fragments of parietal bone and parathyroid gland tissue has been widely ac-

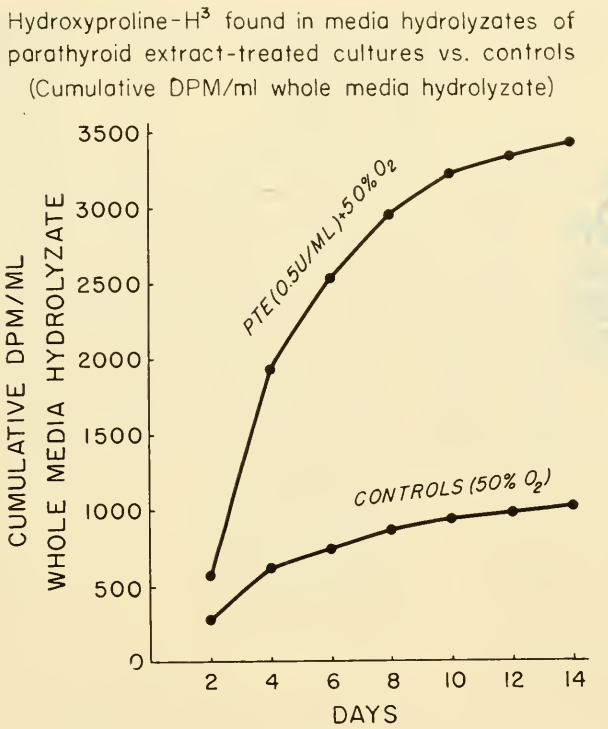


FIG. 28C. Same data as shown in Fig. 28B, but graphed as *cumulative* counts. Note that at the end of 2 weeks the cumulative count of hydroxyproline-H^3 per ml of media hydrolyzate of parathyroid extract–treated cultures totals more than 3 times the value derived from the control cultures. (Data reproduced from Stern *et al.*, 1963*a*, 1963*b*.)

claimed (McLean, 1956), his similar findings of intense bone resorption with crystalline vitamin A and calciferol (Barnicot, 1948) have not received much attention in this country. Although his experiments strongly suggested a direct effect of these agents on bone, clear-cut proof of such a potential can be obtained only in tissue culture. Such evidence has been obtained for parathyroid tissue and extract, as discussed earlier in this paper. Similar evidence of a direct effect of vitamin A on bone in tissue culture has been accumulated during the past decade by Dr. Honor B. Fell and her co-workers. Fell and Mellanby (1952) found that addition of vitamin A acetate or alcohol to the medium resulted in a profound effect on

fetal mouse bones grown in tissue culture. Both the cartilage matrix and the bone were destroyed, the effect being more pronounced the younger the limb-bone rudiments and the higher the concentration of vitamin A. Biochemical evidence was obtained in support of the hypothesis that vitamin A acts on cartilage matrix by enhancing the enzymic activity of chondrocytes and by increasing proteolytic activity through alteration of lysosome permeability (Dingle *et al.*, 1961; Lucy *et al.*, 1961; Dingle, 1961). Of interest was the finding that vitamin A in low concentrations released a protease from isolated lysosomes of rat liver cells (Fell *et al.*, 1961). This *in vitro* effect of vitamin A upon lysosomes has been demonstrated recently in an *in vivo* system by Janoff and McCluskey (1962). These investigators have reported that peritoneal macrophages and polymorphonuclear leucocytes of vitamin A–treated guinea pigs showed a marked loss of acid phosphatase, presumably due to disruption of the intracellular lysosomes which contain the enzyme.

Although Fell and Mellanby (1952) reported the presence of only a few osteoclasts in most of their vitamin A experiments, it would be of interest to learn whether the acid phosphatase content of these cells is depleted.

Attempts to demonstrate a direct effect of vitamin A on mouse calvaria in our own tissue culture system have been successful. In cultures gassed with 50 per cent O_2, the addition of 30 units of crystalline vitamin A alcohol per ml of medium produced bone resorption. The addition of 3 units of vitamin A per ml of medium had less effect, whereas 300 units of vitamin A per ml had the least effect (Fig. 29). It should be noted that our effective dose of approximately 30 units of vitamin A per ml was similar to that found by Fell and Mellanby (1952).

Of further interest was the finding that there was an interdependence between vitamin A and oxygen tension similar to that found for parathyroid extract. From Figs. 30 and 31 it may be seen that vitamin A–treated cultures (24 units per ml) showed no gross effect as compared with untreated controls when both groups were gassed with 10 per cent O_2. Definite enhancement of resorption was obtained with 20 per cent O_2 and vitamin A. The most intensive resorption, comparable to the effect observed with 0.5 unit of para-

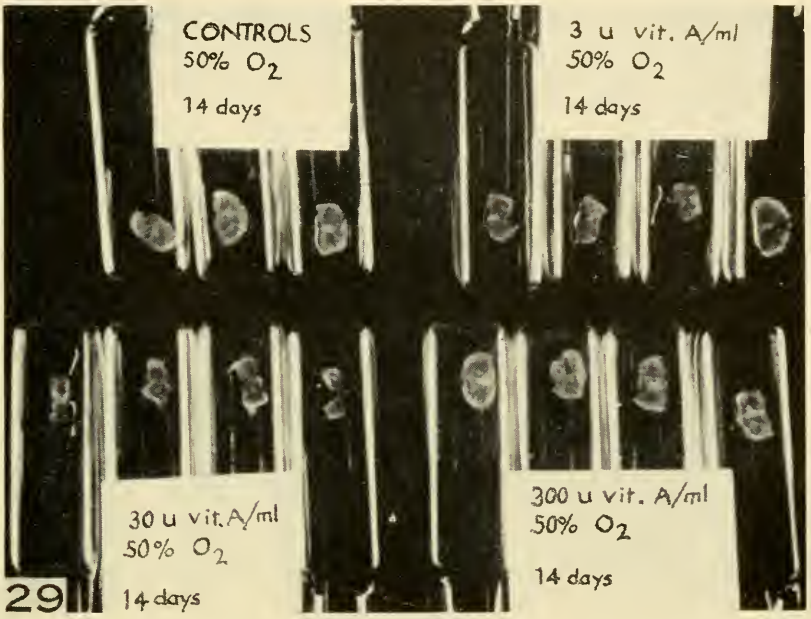


FIG. 29. Gross appearance of 2-week roller-tube cultures exposed to 50 per cent O_2 and various concentrations of crystalline vitamin A alcohol. Although bone resorption was obtained with 3 or 300 units of vitamin A per ml, the greatest effect was found with 30 units per ml.

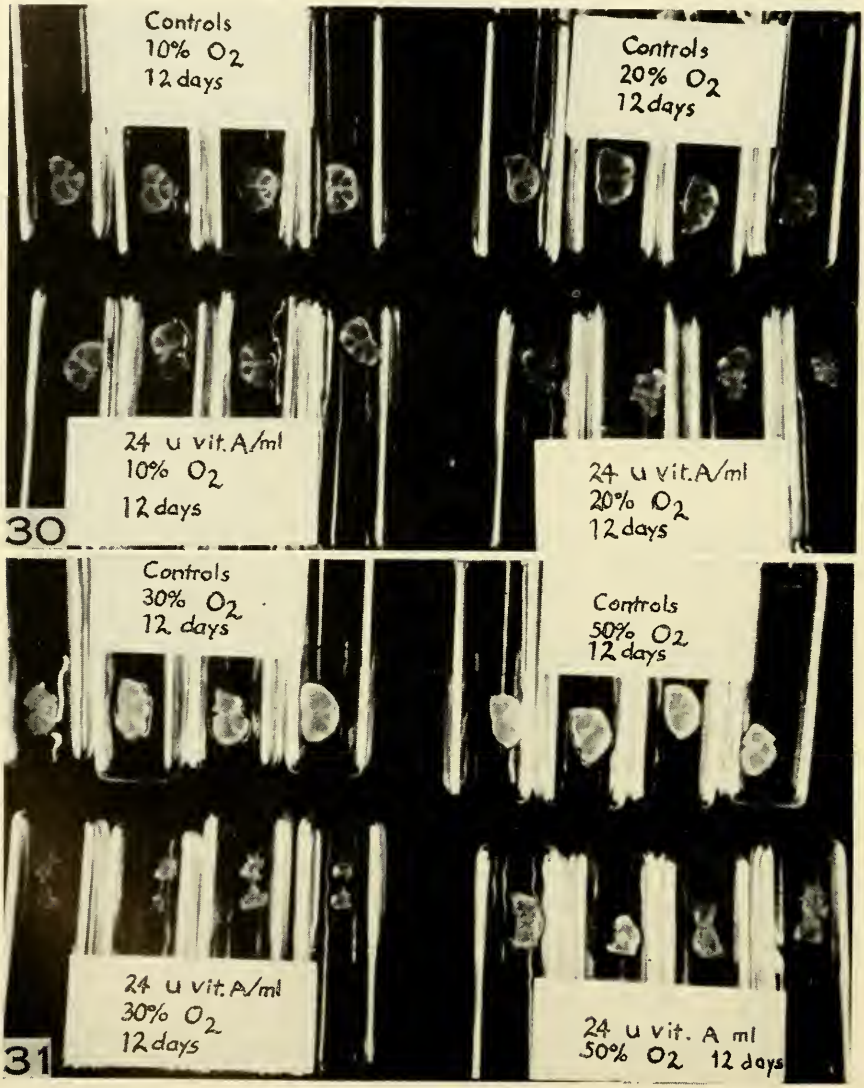
thyroid extract per ml of medium and 50 per cent O_2, was obtained in those tubes containing vitamin A and 30 per cent O_2. Surprisingly, the response to vitamin A and 50 per cent O_2 was not so marked, suggesting that the optimal oxygen tension for bone resorption with vitamin A–containing cultures is at a lower level than that for parathyroid extract–containing cultures.

Attempts to demonstrate enhancement of bone resorption in our tissue culture system with β-carotene were unsuccessful, confirming Barnicot's negative results with the vitamin A precursor (Barnicot, 1948).

Effect of Vitamin D

Although one of the major functions of vitamin D is control of calcium absorption from the gut, it is believed that the vitamin can also influence bone resorption (McLean and Urist, 1961). Not only

does this latter function resemble the action of parathyroid hormone, but some investigators have even postulated that parathyroid hormone acts by modifying the action of vitamin D (Neuman and Dowse, 1961).



FIGURES 30 AND 31

In view of the controversy which still exists concerning a direct action of vitamin D on the skeleton, it was deemed worth while to test the effect of this vitamin in our tissue culture system (50 per cent O_2). Dihydrotachysterol, a member of the vitamin D group which is closely related to calciferol and has calcemic activity *in vivo,* caused a clear-cut enhancement of resorption when added to our system. The extent of bone resorption was directly related to the concentration of dihydrotachysterol in the medium. The microscopic appearance of living cultures exposed to 0.001 mg, 0.01 mg, and 0.1 mg of dihydrotachysterol per ml of medium for 1 week may be seen in Figs. 32 to 35. Preliminary experiments with crystalline vitamin D_2 or D_3 in our system (50 per cent O_2) have demonstrated enhancement of bone resorption when the vitamins were added at the level of 0.1 mg (4000 units) per ml to the medium. The extent of resorption, which was similar to that found with 0.1 mg per ml of dihydrotachysterol, was not so marked as that obtained with 0.5 unit of parathyroid extract per ml of medium. Further experiments are continuing with the D vitamins to determine optimum dosages and the influence of oxygen tension on their ability to induce bone resorption.

Discussion

Several points about these findings seem worth emphasizing and discussing briefly.

It should be stressed that variation in culturing techniques or media can make a vast difference in the response obtained to a given

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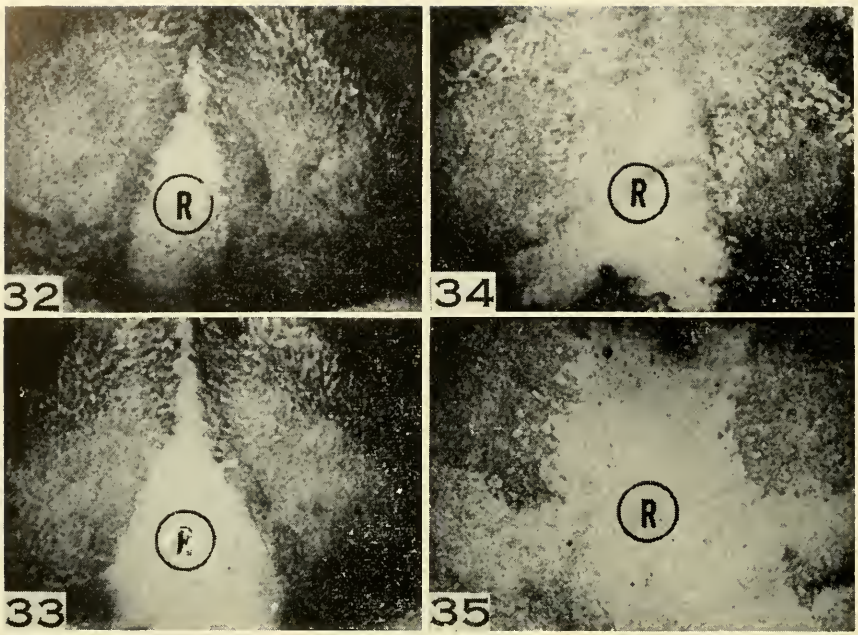
Fig. 30. Gross appearance of 12-day roller-tube cultures containing 24 units vitamin A per ml and exposed to 10 per cent and 20 per cent O_2. Note the enhanced resorption induced by the presence of vitamin A as compared with controls, as well as the influence of 20 per cent O_2 as compared with 10 per cent O_2.

Fig. 31. Gross appearance of 12-day roller-tube cultures containing 24 units vitamin A per ml and exposed to 30 per cent and 50 per cent O_2. Note that the best resorption in the experiment occurs with the combination of 24 units of vitamin A per ml and 30 per cent O_2. Although the vitamin A cultures gassed with 50 per cent O_2 show less of an effect at this stage, it should be noted that this group demonstrated the best resorption microscopically during the first 6 days.



FIGS. 32 TO 35. Microscopic appearance of 7-day roller-tube cultures gassed with 50 per cent O_2 and treated with various concentrations of dihydrotachysterol. Note the increased frontal bone resorption (*R*) as compared with the control (Fig. 32) as the concentration of dihydrotachysterol is raised from 0.001 mg/ml (Fig. 33), to 0.01 mg/ml (Fig. 34), to 0.1 mg/ml (Fig. 35). (All figures × 14.)

test substance. The fact that elimination of embryo extract from our supernatant fluid results in decreased bone resorption in response to oxygen indicates that the "oxygen effect" with our embryo extract–containing medium depends on the simultaneous presence of some unknown bone-resorbing cofactor in the extract. Paradoxically, the elimination of embryo extract permits parathyroid extract, in the presence of oxygen, to stimulate bone resorption, thereby suggesting the presence of a parathyroid inhibitor in embryo extract. This latter possibility is analogous to the findings of Chen (1954) concerning the inhibitory effect of embryo extract on insulin in tissue culture.

Of interest is the finding that the effect of parathyroid extract is directly dependent on the amount of oxygen in the system. This effect of oxygen may be due to a generalized metabolic priming of the target cells, making them more susceptible to the action of bone-

resorbing factors—including parathyroid extract. Perhaps the enhancing effect of oxygen is directly related to increased formation of ATP, since the cells involved in bone resorption are obviously most active and must require large amounts of energy to carry out the complicated task of bone resorption. The inhibition of bone resorption in our system by the addition to the medium of 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, provides experimental evidence favoring this hypothesis. The influence of oxygen tension on vitamin A–induced resorption and probably on vitamin D–induced resorption may be explained on a similar basis. Since the oxygen tensions delivered to the bone-resorbing cells in the calvaria were probably within physiological limits, it is possible that regulation of bone resorption *in vivo* might be accomplished by variation of local oxygen tension through alterations in the local vasculature. Such a scheme would allow local control or modification (enhancement or inhibition) of the effects of a variety of systemically circulating bone resorption stimulators.

The finding that several D vitamins (dihydrotachysterol, crystalline vitamin D_2 or D_3) exhibit marked enhancement of bone resorption in our tissue culture system provides additional evidence favoring the concept that vitamin D may have a similar direct effect on bone *in vivo*.

Although enhanced bone resorption was demonstrated in our system by the addition of either parathyroid extract, crystalline vitamin A, dihydrotachysterol, or crystalline vitamin D_2 or D_3, the difference in results obtained with such closely related compounds as β-carotene and vitamin A emphasizes the specificity of the stimulation. How these compounds alter the metabolism of bone cells must be the subject of continued experimentation.

Summary

1. The extent of bone resorption can be regulated in roller-tube cultures containing chick embryo extract by variation of the oxygen tension in the gas phase.

2. Parathyroid extract, when introduced into the supernatant medium from which embryo extract had been eliminated, induced marked and progressive bone resorption which could be detected

grossly. Control tubes showed minimal resorption despite the presence of adequate oxygen tensions.

3. An interrelation between oxygen tension and parathyroid extract could be demonstrated. Alteration of either factor affected the extent of bone resorption.

4. Biochemical studies performed during the first 4 days of culture on parathyroid-induced resorbing calvaria revealed increased citrate and lactate levels in the media as compared with nonresorbing controls. By the 6th to 8th day of culture, lactate levels (which correlated with glucose utilization) were higher in the nonresorbing control media. The citrate levels remained elevated in the resorbing culture media and paralleled the calcium and phosphate levels.

5. Biochemical analyses of the media of resorbing calvaria containing isotopically labeled collagen revealed significantly more labeled hydroxyproline in the media from parathyroid-treated samples than from the controls.

6. Crystalline vitamin A alcohol (approximately 30 units/ml) produced marked bone resorption in tissue culture. As is the case with parathyroid extract, the effect of vitamin A is dependent on the oxygen tension.

7. Both dihydrotachysterol (0.01 to 0.1 mg/ml) and crystalline vitamin D_2 or D_3 (0.1 mg/ml) significantly enhanced bone resorption in roller-tube cultures gassed with 50 per cent O_2, a finding which supports the concept of a direct action on the skeleton of large doses of vitamin D.

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The Possible Role of Chelation in Decalcification of Biological Systems

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NEUMAN and Neuman (1958) have pointed out in their stimulating monograph the three most reasonable hypotheses to solve the problem of how the calcified tissues may dissolve in the surrounding fluids which are in general believed to be already supersaturated with respect to bone mineral. The most discussed possibility is that the environment of the bone cells is maintained at a lower pH and is therefore locally unsaturated as a result of metabolic acids' leaving the cells. A second possibility is that either the cells or the bone matrix releases a chelating substance which can dissolve the bone salts even in an environment which, on an ionic basis, is saturated or supersaturated. Thirdly, if the cell were surrounded by a high concentration of certain ions which take part in surface exchange (e.g. magnesium, carbonate, or citrate), these ions could increase the apparent solubility of the bone.

The Nature of Chelation and the Chelating Agents Present in Tissues

In addition to forming the familiar salts with metallic ions, certain acids are capable of forming complex ions such as ferricyanides or

platinichlorides. In some cases, owing to the existence of two or more groups in the acid molecule which from their nature and steric position can combine with a metal, the latter becomes part of a ring compound (Fig. 1). Such compounds are called "chelates," and the

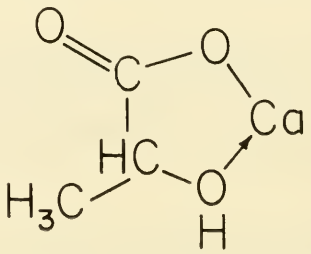

FIG. 1. The structural formula of one of the possible forms of calcium lactate chelate.

acids possessing the necessary groupings are called chelating or sequestering agents. For example, acetic acid combines with calcium to form the salt calcium acetate, but when an amino group is introduced into the molecule (thus converting it into glycine), calcium can be attached both to the acid group (by ionic bonds) and to the amino group (by coordinate covalent bonds), thus forming the ring. Although the term "chelate" should be used only for this special type of complex ion in which the metal is part of a ring, there has been some tendency to use it loosely for complex ions in general. Chelates are characterized by great stability, especially if the metal is attached to the molecule at several sites. Some acids can combine with metals in two ways: as an ordinary salt, and as a chelate. The chelating properties of a substance are greatly influenced by pH and tend to increase as pH rises, because the metal ions compete directly with hydrogen ions. This is of great practical importance because many chelating agents are capable of dissolving at neutral or alkaline pH values salts such as calcium phosphate previously regarded as virtually insoluble except in acids. Chelators may dissolve these salts even in solutions such as blood and saliva which, from the ionic point of view, are already saturated.

Many naturally occurring substances have a structure which enables them to form chelates, and this fact has suggested the possibil-

ity that chelation plays an important part in biological processes. Mandl *et al.* (1952, 1953) and Mandl and Neuberg (1956) have listed the groups of such substances, which include the salts of nucleotides (e.g. adenosine triphosphate), polyphosphates (such as pyrophosphates), phosphorylated sugars, amino acids, and the hydroxy, keto, dicarboxylic, and tricarboxylic acids.

Among the biochemical processes which it has been suggested may occur by chelation are the absorption of insoluble salts from the soil by the root hairs of plants, the transport and deposition of metallic ions (e.g. calcium), and the control of enzyme action by chelation of inhibitors or activators. In the present paper we are concerned with the possible role of chelation in dissolving the hard tissues in tissue fluids already saturated with calcium phosphate.

The Phenomena to Be Explained

Calcified tissues are removed during the physiological processes of bone growth and remodeling, and of resorption of the roots of deciduous teeth, and in meeting a calcium stress such as pregnancy and lactation, especially on a low calcium diet. Pathological processes involving decalcification are dental caries, hyperparathyroidism, and infection of bones, including periapical abscesses. There are no reasons for believing that all these diverse phenomena can be accounted for by one single process.

Although several of the above processes are accompanied by the presence of osteoclasts—and it can no longer be doubted that osteoclasts do take an active part in resorption—it is far from certain that these are the only cells concerned. Apart from the osteolysis described by Bélanger *et al.* in this symposium (chapter 20), several workers (Woods and Armstrong, 1956; Talmage and Elliott, 1958) have reported that in experimental hyperparathyroidism resorption occurs from bone deposited several months previously which is inaccessible to osteoclasts. Heller-Steinberg (1951) reported histological changes, presumably related to bone dissolution, in the matrix surrounding the lacunae containing osteocytes. A photomicrograph by Raisz *et al.* (1961) also showed that parathyroid hormone treatment produced resorption in the neighborhood of osteocytes and

that osteoclasts were not present. The strong implication of all this work is that osteocytes can take part in resorption.

The Unique Position of Citrate

As Neuman and Neuman (1958) pointed out, citrate is a unique ion in that it can act in all the three ways in which they suggested bone could be dissolved in tissue fluid. It is an acid with three carboxylic groups, it forms a stable chelate with calcium, and it can exchange with the phosphate of apatite to form a more soluble crystal. The idea that bone dissolves by the local formation of citrate is therefore very attractive. The evidence which has accumulated during the past decade or so favoring the view that citrate is important in bone resorption has been so well reviewed elsewhere (Neuman and Neuman, 1958; Freeman, 1960) that it need not be repeated here. Neuman and Neuman also proposed a hypothesis relating the actions of vitamin D and parathyroid hormone to citrate metabolism. They suggested that vitamin D promotes citrate synthesis, perhaps by activating an enzyme cofactor, e.g. DPN (NAD), whereas parathyroid hormone might prevent oxidation of citrate by inhibiting TPN (NADP)–linked reactions.

In the past two or three years, however, it has become clear from *in vitro* studies of bone metabolism that lactate and not citrate is the major end product of bone, a finding which has cast doubt on the citrate theory (Borle *et al.*, 1960*a*, 1960*b*). Although lactate is an α-hydroxy acid and might be expected to have chelating properties, as some workers have, in fact, reported (Johnston, 1956), recent opinion is that its chelating action on apatite crystals is negligible (Leach and Dodge, 1961; Gray *et al.*, 1961). If lactate dissolves bone *in vivo*, then it does so as an acid rather than as a chelator.

Citrate versus Lactate: Recent Evidence

Although it is tempting to reject the citrate theory and with it reject the main support for the idea that chelation is important in hard tissue destruction, such a rejection is probably premature.

It might be thought that one way of testing whether citrate or lactate is the bone solvent would be to find whether bone resorption

is invariably accompanied by an increase in citrate or lactate production. This has been attempted by various groups of workers, but their results are contradictory. One practical reason for this may be the analytical difficulties of detecting changes in the very small absolute amounts of citrate which are produced. This approach may be unsound, however, because if both osteoblasts and osteoclasts are concerned in bone removal, they may act by different mechanisms, and there may not be one uniform metabolic product invariably associated with bone removal.

Even if citrate production is very low in relation to lactate production, it is still possible to envisage mechanisms by which citrate might be of importance. We might consider the following possibilities: (1) that citrate production of bone may occur mainly in areas of resorption; (2) that small changes in citrate concentration may, because of its special properties, have quite large effects on bone solubility; (3) that changes in citrate production by bone cells may alter the citrate concentration and solubility properties of the bone crystal.

Hypothesis 1. In most bone samples, osteoblasts greatly outnumber osteoclasts, so that if, as might be speculated, the former produced lactate and the latter citrate, there might be very high local concentrations of citrate in the vicinity of, or within, osteoclasts in areas of resorption, but very little citrate in the surrounding medium.

Few of the workers who have studied the metabolism of bone *in vitro* have reported on the relative numbers of osteoblasts and osteoclasts in the tissues they incubated, so that it is impossible to test this hypothesis rigorously with existing data.

Many workers have, however, found an increased citrate production by bone after parathyroid treatment, which would be expected to increase the number of osteoclasts. It is also of interest that Raisz *et al.* (1961) showed that pretreatment with parathyroid extract (PTE) increased the amount of calcium dissolving *in vitro* from incubated bone without an increase in osteoclasts. This presumably osteoblastic resorption was not accompanied by any change in citrate formation, but lactate production did increase under anaerobic conditions. One piece of evidence against the citrate theory was pre-

sented by Vaes and Nichols (1961). They found that the amount of calcium dissolving *in vitro* from PTE-treated bone was higher than from controls even in a medium containing 1.5 mM citrate, i.e. so high a level that the small addition from the cells would not be expected to have any influence. The cells might, however, be producing such high concentrations in their immediate vicinity that the citrate was still active. The probability that this occurred was increased somewhat by the finding that the absolute amounts of bone dissolving from the control samples were no greater in the citrate buffer than in the other media, suggesting that, for some reason, the citrate buffer was not dissolving more than other buffers.

Recent work on the fundamental action of parathyroid hormone (PTH) has emphasized the possibility that one of its effects may be on an individual enzyme system. Could not its action be a stage farther back than this, and is there not evidence that one of its fundamental actions is to stimulate osteoclast formation? If these cells produce citrate, as is speculated here, then their formation would account for the increased citrate production by bone without involving reactions at the enzyme level (presumably the formation of osteoclasts is more complicated than an interaction with one enzyme system). It is realized that this cannot be the only effect in bone, since, as already mentioned, PTH seems also to bring about resorption by osteocytes.

Hypothesis 2. In view of the special powers of citrate in making bone soluble, it seems quite reasonable to suggest that although the citrate is only 1 to 2 per cent of the total acid produced by bone, it may have a much higher proportional effect on dissolving bone. This is a difficult point to test *in vivo*, but unpublished *in vitro* tests have shown that adding citrate to molar lactic acid buffer so that the citrate/lactate ratio is 1/100 may increase the amount of calcium phosphate dissolving by as much as 5 to 10 per cent. Anomalous results are obtained with some other buffers, however.

If this hypothesis is substantiated, this could be a means by which citrate plays an important part in controlling bone solubility even if citric and lactic acids are both formed by the same cells.

Hypothesis 3. There is good evidence that citrate can exchange with the phosphate in hydroxyapatite and that this is associated with an increased solubility of the crystals.

Schartum and Nichols (1961) concluded that much of the effect of pretreatment with PTE on the dissolving of bone *in vitro* could be accounted for by changes in passive solubility rather than by effects on cell metabolism, since it could be detected in bone inactivated by heat. They suggest that the increased citrate concentration in bone found by others after parathyroid treatment might account for this change in solubility. In a later paper Schartum and Nichols (1962) showed that if the solubilities of living bone and bone killed by heating were compared in media over a range of pH values, the living bone dissolved more readily above pH 6.5, but below this pH both samples dissolved to the same extent. This could mean that living bone maintains the fluid bathing the crystal surface at a pH of about 6.5, so that if the medium was at that pH already the living bone had no means of increasing solubility; this would favor the idea that acid is the solubilizing agent. These results were in good agreement with the conclusion of Nordin (1957) that plasma is in equilibrium with bone if the pH on the surface of the crystal is about 6.6 to 6.8. Schartum and Nichols were reluctant to conclude that their results supported the acid theory, since, as they pointed out, at a low pH value some mechanisms normally at work, such as citrate production, might be impaired.

Hartles (1961) has summarized the results from his own laboratory on the factors influencing the concentration of citrate in bone. Briefly, he reports that diets deficient in vitamin D and calcium led to the formation of bone low in calcium and citrate, and that the animals developed tetany. With the same low calcium diet to which normal or high levels of vitamin D were added, tetany did not occur despite the fact that the concentration of calcium in the bone was not significantly altered, although the bone citrate was much higher (Table I). Hartles and Leaver (1961) point out that the bone with the higher citrate appeared to be able to supply calcium to the blood (presumably in response to the increased parathyroid stimulus as a result of the low calcium diet) and thus avoid tetany, whereas the

TABLE I. Composition of Rat Femurs on Various Diets
(From Hartles and Leaver, 1961)

	Ca (%)	P (%)	Citric acid (%)	
Control	24.3	12.1	0.51	
Low calcium	19.7	9.9	0.76	No tetany
Low vitamin D	23.5	11.6	0.46	
Low calcium and low vitamin D	19.9	9.7	0.24	Tetany

Conclusion: Calcium readily mobilized from bone with high citrate but not from bone with low citrate.

bone with the low citrate was unable to supply sufficient calcium to avoid tetany. Studies on the extraction of citrate from bone show that some fractions of it are more readily dissolved out than others, and Hartles and Leaver tentatively suggest that some of it may be regarded as a "constant fraction" and some as a "variable fraction." This work suggests that one of the functions of vitamin D is to increase the citrate in the variable fraction and make the calcium available to parathyroid influence. How this happens is not known, but it might be speculated that the simultaneous presence in bone of the citrate and calcium facilitates the release of a calcium citrate complex. The suggestion is, then, that the citrate in the bone, rather than citrate newly formed by bone cells, is an important factor in calcium mobilization.

Hartles and Leaver seem to have overlooked another possible explanation of their results, namely, that vitamin D prevented tetany by increasing the absorption of calcium from the low concentrations in the diet and digestive juices.

Incidentally, Hartles and Leaver point out that their results with diets containing normal levels of vitamin D but low in calcium supported Neuman and Neuman's hypothesis on vitamin D action in that the bone citrate was raised under conditions which would be expected to stimulate the parathyroid. In the groups without vitamin D, however, the hypothesis would suggest that with both calcium and vitamin D low in the diet (i.e. with parathyroid stimulated), the citrate should be higher than in a diet with normal calcium and low in vitamin D. The reverse was found.

The Dissolving of Apatite within Osteoclasts

Four groups of workers (Cameron and Robinson, 1958; Hancox and Boothroyd, 1961; Gonzales and Karnovsky, 1961; Scott and Pease, 1956) have now demonstrated, in electron micrographs, bone salt crystals within the osteoclast. The crystals enter by pinocytosis in the "ruffled border" and can be seen in the cytoplasmic vacuoles. There is at present no means of knowing whether *all* the crystals removed by osteoclasts enter the cell. The crystals presumably dissolve in the cytoplasmic vacuoles and the dissolved products are shed into the tissue fluid. The question now becomes: What is the nature of the *intracellular* mechanism for dissolving the crystals and discharging the salts in a form in which they will remain soluble in the tissue fluids? The contents of cells are stated to be acid (Caldwell, 1954); much of the intracellular calcium is likely to be bound to protein, and the phosphate is also largely organically bound. These conditions of acidity and low concentrations of calcium and phosphate ions would favor the dissolving of the crystals perhaps without the intervention of chelating substances. However, since the osteoclasts are presumably taking in many apatite crystals, the capacity for binding the calcium and phosphate would become rapidly used up—unless the organization of the cell can prevent the contents of the vacuoles from having access to the protein and enzymes of the cytoplasm. The probability is, then, that the cell needs chelating agents to dissolve the crystals as well as to discharge the calcium into the circulation in a form in which it will remain soluble.

Possible Importance of Chelating Agents Released from the Matrix

There remains the problem of how the crystals are dislodged from the bone matrix. The electron micrographs of Hancox and Boothroyd showed naked collagen fibers, from which they concluded that the removal of the apatite crystals occurs first, and is followed by the disintegration of the matrix. An extracellular solvent, perhaps citrate or lactate, may dissolve some of the crystals and thus expose the matrix. Scott and Pease (1956) and Gonzales and Karnovsky (1961), on the other hand, saw no matrix in their micrographs and

concluded that the collagen was broken down first, leading to the release of the crystals.

Hancox and Boothroyd, in this symposium (chapter 18), have shown that detached collagen enters the osteoclasts, where it is presumably broken down into products which might be important in chelating the apatite crystals.

Greulich (1961) showed by ultrasoft microradiography that the active osteoclast is surrounded by a band of organic matter 1.0 to 1.5 microns wide. The nature of this material is uncertain, but it could contain chelating agents which might be concerned in the dissolution of the crystals.

Johnston *et al.* (1961) reported a large reduction in the hexosamine content of bone following PTE injections in rats, implying that breakdown of some constituents of the matrix was taking place. The products might play a part in chelating the calcium.

Cretin (1951), in a paper giving few details and no photomicrographs, stated that in freehand sections of unfixed bone, indicators showed that the vicinity of osteoclasts was acid up to a distance of 20 to 30 microns from the cell. This work would be worth repeating, as it might help to decide which cells, if any, were producing acid even though it would not distinguish between citric, lactic, and other acids.

The results of Dulce and Siegmund (1960) and Siegmund and Dulce (1960) are also of interest. They found that inhibition of carbonic anhydrase by Diamox caused a fall in the calcium of blood plasma in laying hens. The explanation they suggested was that inhibition of carbonic anhydrase by Diamox prevented acid secretion and thus bone resorption by osteoclasts. This suggests that carbonic acid might be important, which is support for the views of Forscher and Cohn in this symposium (chapter 22).

The Dissolution of Infected Bone

Very little is known about the means by which infected bone is removed. The presence of pus and inflammation will increase pressure locally, which is probably a stimulus to osteoclast formation (Hancox, 1956), and if so, the problem becomes the same as that of osteoclastic removal of bone elsewhere. Dubos (1955) summarizes

the evidence that areas of inflammation are usually acid, although he states that when infection is present, acidity may not develop owing to damage to the acid-producing cells by the bacteria. The intense autolytic activity in pus might be expected to release the acid contents of cells as well as many substances with chelating properties. It seems possible that pus might have a direct solvent action on bone and not be dependent on osteoclasis.

As few data could be found on the chemistry of pus, a few preliminary observations have been made. Five samples have been collected with pH values ranging between 6.1 and 6.7, and lactate estimations suggested that this was the main acid. It has not yet been possible to obtain pus from infected bone.

These very limited data suggest tentatively that acidity brought about largely by lactate (presumably released from autolyzing cells or formed from their contents) is probably one factor in the removal of infected bone. It is realized that study of pH alone cannot decide the importance of acid. It is essential to know the concentrations of calcium and phosphate ions in order to decide whether, even under acid conditions, the environment of the bone is or is not saturated with bone salt.

Chelation as a Mechanism for Demineralization by Muricid Boring Gastropods

In this symposium (chapter 3) Carriker *et al.* showed that an alkaline buffer extract of the secretory gland associated with the boring mechanism of a muricid boring gastropod was capable of etching oyster shells. As this extract was alkaline, the most reasonable explanation of the etching is that a chelating agent is involved.

Chelation and Proteolysis-Chelation Theories of Dental Caries

Martin *et al.* (1954) pointed out that many substances known to occur in the mouth have chelating properties, and suggested that chelation under neutral or alkaline conditions might therefore be the cause of dental caries. After further consideration of possible sources of chelators, Schatz and Martin (1955, 1962) proposed that

proteolytic bacteria might attack the proteins of enamel, releasing from them various breakdown products which would then form chelation compounds with the mineral matter of the enamel (the proteolysis-chelation theory). Then the organic and mineral phases of enamel would be destroyed more or less simultaneously. This approach is a direct challenge to the acid theory, which in its modern form is based on results which are interpreted as showing that tooth substance does not dissolve in saliva or plaque until acids have lowered the pH of the environment to a critical value, usually between 5 and 6.

As long ago as 1949, Eggers Lura suggested a nonacid decalcification theory of caries, and although he has modified his views he still believes that chelators arising in the plaque are more important than acids (Eggers Lura, 1949, 1957, 1961).

Detailed critiques of these theories have recently been published (Bibby *et al.*, 1958; Jenkins, 1961), and only the main points will be discussed here.

Evidence Needed to Prove the Proteolysis-Chelation Theory

Before the proteolysis-chelation theory is accepted, experimental evidence must provide positive answers to the following questions: Do bacteria exist in the mouth which can attack the organic matter of enamel without previous decalcification? Are the products of this breakdown chelating agents? If so, are the amounts of chelators formed adequate to dissolve the inorganic matter to form a carious cavity?

THE EXISTENCE OF BACTERIA WHICH CAN ATTACK UNDECALCIFIED ENAMEL. No study appears to have been made of the effect of bacteria on isolated enamel protein, presumably owing to the difficulty of obtaining this material. On the assumption (not supported by the analyses of Stack, 1955) that enamel protein is a keratin, Schatz *et al.* (1957, 1958) cultured oral bacteria in media containing keratin from carefully washed hair, wool, and feathers as the only source of nitrogen. They reported that both human and animal saliva contained bacteria which grew on these media, and considered that the existence of oral keratinolytic bacteria was thereby proved. This

conclusion was criticized by Mandel and Ellison (1957) on the ground that the bacteria may not have been metabolizing the keratin, but various nitrogenous contaminants. The thorough washing to which these sources of keratin were subjected makes this unlikely, although Schatz *et al.* did not prove conclusively that all non-keratin nitrogenous substances had been removed.

Whether we accept or reject this evidence for the presence of oral keratinolytic bacteria is probably unimportant because the majority of the enamel protein is not keratin, but mucoprotein and polypeptides (Stack, 1955). There is little doubt that some oral bacteria could attack these substances.

The question whether enamel organic matter can be attacked without previous removal of the calcium salts is more controversial. Most of the evidence suggests that the calcium salts, both in enamel and in dentine, protect the protein from enzyme attack (Miller, 1890; Bibby, 1932; Evans and Prophet, 1950; Roth, 1957), but some previous workers have disagreed (Pincus, 1937; Hurst *et al.*, 1953). Schatz *et al.* (1955) measured the oxygen uptake by cultures of oral bacteria in the presence of whole teeth, hair, rat and human dentine, and human enamel (Fig. 2). Respiration increased with every addition, especially with the human teeth and rat dentine. With human enamel, the rise in oxygen uptake was extremely small and of doubtful significance, although in later publications larger effects from human enamel were reported (Fig. 3). These results were interpreted as showing that the organic matter in intact enamel could be metabolized by oral bacteria.

Evidence for the chelating action of the substances released from incubated hard tissues. In another series of experiments by Schatz *et al.* (1955), bone containing Ca^{45} was incubated with and without oral proteolytic bacteria and the rises in radioactivity of the media were compared. In the presence of the bacteria 77 per cent more Ca^{45} entered the medium (pH 8.4) than in the control medium which remained at pH 7.0, from which it was concluded not only that the bacteria could attack the undecalcified bone, but also that the products of proteolysis dissolved the calcium by chelation under these alkaline conditions.

FIG. 2. Respiration of a strain of proteolytic bacteria isolated from a human mouth and later grown in the presence of autoclaved human tooth (1), untreated human tooth (2), hair (3), rat molar dentine (4), rat incisor dentine (5), human dentine (6), human enamel (7), no addition (autorespiration) (8). (Courtesy of Dr. Albert Schatz and the *New York State Dental Journal.*)

Experiments on somewhat similar lines were carried out by Hashimoto (1958, 1960), who incubated cow and human teeth with bacteria and detected a rise in concentration of nonprotein nitrogen, calcium, magnesium, and phosphate in the medium. The parallelism between the rise in nonprotein nitrogen and in mineral matter was interpreted as evidence for proteolysis followed by chelation of the metallic ions by the products of proteolysis.

Although these conclusions are quite reasonable, they are by no means the only possible explanation of the results, and in any case their relevance to caries is speculative. In particular, the media in which these incubations were carried out were not saturated with calcium phosphate, unlike saliva at neutrality and probably plaque at neutrality (Dawes and Jenkins, 1962). It is therefore very probable that at least some of the mineral matter was dissolved in the media without involving chelation, and exposed the organic matrix, which was then attacked by bacteria. These experiments did not,

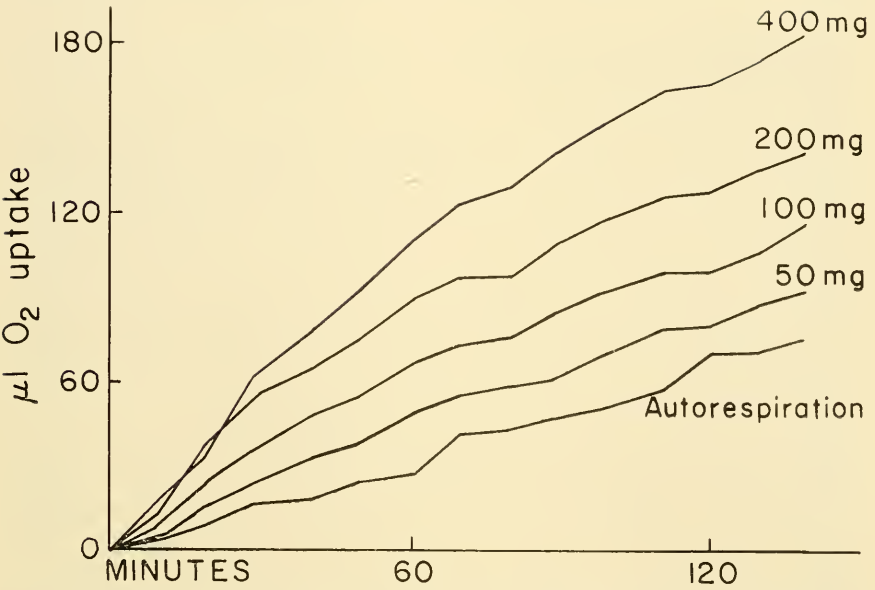

FIG. 3. Respiration of proteolytic bacteria isolated from a human mouth and grown in the presence of different amounts of human enamel. (Courtesy of Dr. Albert Schatz and the *New York State Dental Journal.*)

therefore, prove that the organic matter of the hard tissues was attacked before decalcification, nor that the mineral matter had been dissolved by chelation. Also, in some of the experiments (especially with ground enamel and dentine), some of the organic matter is quite freely soluble (Stack, 1955; Evans and Prophet, 1950), and its release does not necessarily prove proteolytic action. There is no reason to doubt that many of these products of proteolysis are chelating agents. It is, however, impossible to decide how effective these are or what proportion of the mineral matter which dissolved did so by chelation. Other workers whose results have been quoted in support of the proteolysis-chelation theory of caries are Saito (1957), Stüben (1959), Wandelt (1959), and Eggers Lura (1949, 1957, 1961). In our view all their results are inconclusive as far as caries is concerned, either because their experiments were inadequately controlled (Wandelt and Eggers Lura) or because they did not reproduce conditions of saturation with calcium phosphate which resembled those in saliva.

A Criticism of Experiments on the "Critical pH" and Its Investigation

Since saliva is saturated at neutrality with calcium and phosphate, tooth substance would be expected to be insoluble. If the pH falls steadily, it will eventually reach a value at which the calcium and phosphate concentrations are no longer able to saturate the saliva, and below this value tooth substance would be expected to dissolve. After the addition of chelating agents, however, apatite would dissolve even in saliva which, on an ionic basis, was previously saturated. In other words, the concept of a critical pH would be invalid. Experiments designed to test the hypothesis of a critical pH have consisted of measuring the solubility of tooth substance either in saliva at different pH values (Fosdick and Starke, 1939; Ericsson, 1949; Hills and Sullivan, 1958) or in buffers saturated with calcium phosphate over a pH range (Enright *et al.*, 1932; Hills and Sullivan, 1958). In the saliva experiments, toluene or thymol was added to prevent bacterial activity from making the pH unstable, but it was not realized that this would also prevent the formation of chelators either from saliva constituents or from enamel.

It seemed to us desirable, therefore, to find out whether mineral matter from enamel could dissolve in saliva incubated without carbohydrates or toluene. Such a saliva would become alkaline, and the pH would be so much higher than the critical value that no apatite could dissolve unless chelating agents were formed. Over forty experiments were carried out, and the results (Table II) showed that

TABLE II. Calcium and Phosphate Concentrations (μG ML) before and after Incubating Salivary Sediment with Teeth for 48 Hours

	Before incubation	After incubation		Difference (teeth − control)
		Control	With teeth	
pH	—	7.8	7.9	+0.1
Calcium	—	90	88	−2
Inorganic phosphorus	170	187	206	+19
Total phosphorus	280	280	280	0

although the concentration of dissolved calcium did not change during the incubation of teeth with saliva (or saliva sediment), there was a fairly consistent rise in the inorganic phosphate as compared with control media containing saliva but no teeth (Jenkins, 1959; Jenkins and Dawes, 1963). To determine whether this was a loss of phosphate from the tooth or a breakdown of organic phosphates in saliva, total phosphate (i.e. organic plus inorganic) was estimated in many of the experiments, and in most cases it showed no change. It was concluded that the presence of the tooth surface favored the breakdown of organic phosphate in the same way as White and Hess (1956) had found that ashed bone appeared to act as though it contained phosphatase. The explanation of the few experiments in which total phosphate in solution did rise (again with no evidence of a rise in calcium) is not clear; but possibly adsorbed phosphate was released. How it entered a medium already saturated with calcium phosphate and why it should be released in some experiments and not others is unexplained. These experiments provide no evidence for the formation of chelating agents either from salivary constituents or from enamel. It is possible, of course, that chelators might have formed but in too low a concentration to dissolve detectable quantities of calcium. In any case, this negative evidence in no way disproves the proteolysis-chelation theory, but merely makes it somewhat less probable.

Quantitative Considerations

An obvious difficulty of proteolysis-chelation as a mechanism of enamel caries arises from the low concentration of organic matter in enamel. Could 0.6 per cent of organic matter (excluding lipid), even if it were broken down as Schatz suggests and even if the whole of it had chelating properties, dissolve the 99.4 per cent of mineral matter? Bibby *et al.* (1958) considered this to be one insuperable difficulty in the theory. It has been answered by Schatz *et al.* in several ways. First, they speculate that small areas of destruction of enamel may open up pathways through which other chelators from plaque or food can enter and extend the cavity. Secondly, they have calculated from the amount of bone dissolved during 2 hours in their *in vitro* experiments that 11 mg of enamel

could be dissolved by chelation in a year. They assumed that the rate of decalcification in a cavity *in vivo* was the same as in their experiments *in vitro*, allowing for the much lower protein content of enamel as compared with bone. Though fully realizing the limitations of this calculation, Schatz *et al.* at least think that it shows that chelation could have effects of the right order of magnitude. However, the rate *in vivo* is very unlikely to be as rapid or as constant as that *in vitro*, and the whole basis of the calculation is therefore unsound.

In conclusion, the proteolysis-chelation theory is original and stimulating, but most of the experiments so far carried out to answer the questions raised above are either negative or susceptible to alternative explanations, or do not resemble sufficiently the conditions in carious cavities to be relevant. When this evidence is contrasted with the mass of evidence on the acid theory, which—though mostly circumstantial and inconclusive—so largely favors the theory, the probabilities seem against proteolysis-chelation in the form so far discussed.

Possible Chelators in Plaque

The original chelation theory of Schatz *et al.* (1955), which did not confine the source of chelators to the organic matter of enamel, is more plausible than the later proteolysis-chelation theory. It cannot be denied that many substances in foods, saliva, and plaque possess chelating powers. The question that arises is whether the concentrations are sufficiently high to decalcify enamel, and in particular whether their action is quantitatively comparable with that of acids. Plaque is known to have a high calcium concentration (Allen and Moore, 1957; Dawes and Jenkins, 1962), and it is possible that much of it is present in a chelate or complexed form. If so, the chelators may be already bound to so much calcium that they are unlikely to combine with the much less soluble surface of the enamel.

Eggers Lura (1960) has tested the effects of certain chelating agents dissolved in ammonia buffer at pH 7.8 on the enamel surface. He stated that alkaline solutions of nucleic acid, PTE, salivary mucin, and amino acids produced signs of decalcification, but the experi-

ments were inadequately controlled, and, since the media were not saturated with calcium phosphate, some solution of tooth substance would be expected in these mixtures by ordinary ionic equilibria without involving complex formation. Sucrose, ATP and other organic phosphates, and sialic acid were also suggested by Eggers Lura as possible chelating agents. Chelation by sucrose is of particular interest because it provides a possible explanation, on the chelation theory, of the undoubted fact that the ingestion of much sucrose increases caries. Blackwell *et al.* (1961) have shown, however, that high unphysiological pH values are necessary for sucrose to attack the enamel surface. ATP and most other organic phosphates of plaque are present inside the bacterial cells and out of contact with the enamel surface. If these substances were released from autolyzing dead cells, much of the organic phosphate would be broken down by the enzymes of the plaque. These substances, although powerful chelators, would not therefore seem to be present under conditions in which they could have much effect on the enamel. The position of sialic acid will be discussed below.

Tests on the Chelating Powers of Plaque Extracts

In an attempt to test the effectiveness of the chelators in plaque, we have made salivary extracts of plaque pooled from over a dozen subjects and shaken them with a piece of bone containing Ca^{45} (Jenkins and Dawes, 1963). Control media contained a similar piece of bone shaken with saliva supernatant only. The radioactivity entering the two media—salivary supernatant alone and saliva supernatant containing any soluble chelating agents of plaque—was identical and could be accounted for by ionic exchange, due allowance being made for variations in surface area of the bone samples by prior incubation of both bone samples with salivary supernatant alone. These experiments were carried out four times in all, and lent no support to the suggestion that alkaline plaque contained effective concentrations of chelating agents. Admittedly, the plaque constituents were diluted sixfold in making the extracts, but this is probably compensated for by the great sensitivity of the radioactive method of detecting whether the bone dissolved. We should like to emphasize the importance of studying *salivary* extracts of

plaque. A very different, and we believe a misleading, result might have been obtained if aqueous extracts of plaque had been used. The crucial issue which we believe needs testing is whether chelators are active in a salivary medium saturated with calcium phosphate.

The Concentration of Certain Chelating Agents in Plaque

The concentrations in plaque of several substances which have been suggested as chelating agents have also been investigated. Citrate concentrations were estimated in plaque collected before and after a glucose rinse, and the rate of breakdown of citrate added to plaque was determined. The results (Table III) show

TABLE III. Citrate and Lactate Concentrations in Dental Plaque

	No. of estimates	μg/100 mg wet plaque
Citrate before glucose rinse	10	7.3
Citrate after glucose rinse	18	3.5
Rate of breakdown of 10 μg of added citrate		
At pH 6.8	30	8.5/min.
At pH 5.0	12	4.9/min.
Lactate (pH above 7.0)	60+	60–270

that the concentrations were extremely low; they are, in fact, near the limit of detection by the method. The concentration after the glucose rinse was significantly ($p < 0.05$) lower than before, which is surprising, as it was expected that carbohydrate metabolism would have increased the citrate concentration. The rapid rate of breakdown of added citrate presumably explains why plaque citrate is at too low a concentration to act as a chelator.

Lactate concentrations in over 60 plaques whose pH exceeded 7.0 ranged from 0.06 to 0.27 mg/100 mg wet plaque. These figures are in fair agreement with those of Moore *et al.* (1956). Leach and Dodge (1961) found that as high a concentration as 0.9 per cent lactate exerted no detectable chelating action, from which it may be concluded that the much lower concentration of lactate in neutral plaque is of no importance as a chelating agent.

The sialic acid concentration of plaque was also found to be very low (averaging 3.7 μg/100 mg wet plaque), and, as with citrate, plaque rapidly metabolized added sialate.

None of this evidence from the experiments on plaque extracts and from the concentration of individual substances reported to have chelating properties supports the hypothesis that chelation is important in caries.

Conclusion

The position may be summarized as follows: There is no evidence which clearly supports the hypothesis that decalcification in caries results from chelators in the plaque. The evidence for acids is very considerable, in spite of uncertainties about the pH on the inner side of the plaque or about the critical pH necessary for decalcification in that area. On the other hand, very few experiments have been carried out on the action of plaque chelators.

Since it cannot be denied that some chelating agents are present in the plaque, it is possible that they may be active during the intervals between meals and during sleep, when the plaque is in the neutral or alkaline conditions which favor chelation. The experiments reported here suggest that such activity must be weak and probably unimportant as compared with the action of acid. More experiments are needed on the quantitative effects and distribution within the plaque both of chelators and of acids before a final decision can be reached.

There are no grounds for believing that chelators are alone responsible for caries. There is no experimental evidence for the view that enamel protein is a source of chelators in caries. The low concentration of organic matter in enamel is a weighty objection to the theory, and the answers given to this objection are purely speculative.

Summary

1. The nature of chelation and its possible biological importance are described.

2. The unique solubilizing powers of citrate are pointed out, al-

though the low concentration of citrate as compared with lactate produced by bone *in vitro* tends to minimize its importance.

3. It is speculated that even small amounts of citrate might be important in bone if they were localized in sites of resorption such as those within or in the immediate vicinity of the osteoclasts. Evidence is reviewed that citrate may enter the bone crystal and increase its solubility.

4. The suggestions on the possible role of chelation in dental caries made by Schatz *et al.* are described and the evidence for them is critically reviewed.

5. It is concluded that, although there are gaps in the chain of evidence for both the acid and the chelation theories, most of the evidence supports acid as the means of decalcification in caries.

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Animal Collagenase and Collagen Metabolism

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TISSUE remodeling during growth and development requires that synthesis and removal of structural elements be precisely synchronized in time and space. Cell migration, aggregation, differential multiplication, and selectively timed and placed cell death are major features of the process. The resultant complexities of the complete tissues have made it difficult to dissect and analyze the relevant mechanisms involved in these processes.

Because of the ubiquitous distribution of the extracellular structural element collagen, because of the singular and specific characteristics of this protein, and because of the large body of knowledge concerning its structure, chemistry, and metabolism, we have chosen to follow its fate in a controllable remodeling system, the metamorphosing tadpole. Metamorphosis can be initiated by thyroid compounds, producing rapid resorption of large collagen-containing structures such as tail fin and gill (Gudernatsch, 1912; Allen, 1929; Frieden, 1961). Simultaneously, there is hypertrophy of other collagenous tissues such as the skin of the back. Local intricate changes are induced in the shape of specialized structures such as the mouth parts, opercular areas, limbs, eye positions, etc.

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The rapid removal of collagen which occurs during bone growth and during retrogression of the postpartum uterus has stimulated an intensive search for an animal collagenase operating under physiologic conditions (Morrione and Seifter, 1962; Woessner, 1962; Rouiller, 1961). The many examinations of tissue extracts for collagenolytic activity operating at physiologic pH and temperature have uniformly been unsuccessful (Mandl, 1961). By culturing tissues on reconstituted collagen gels we have recently demonstrated the appearance of a collagenolytic enzyme in amphibian connective tissues (Gross and Lapiere, 1962). Similarly, we have also detected collagenolytic activity in rat uterine tissue and in bone.

These studies have been extended and we wish to discuss the results of simultaneous experiments on the metabolic turnover of collagen and collagenolytic activity in resting and metamorphosing bullfrog tadpoles.

We have compared the collagen of the tail fin with that of back skin since in the former complete removal occurs during metamorphosis, whereas in the latter there is increased production.

Gross Changes in Tail Fin and Back Skin Connective Tissue in Metamorphosis

The dramatic remodeling of the frog tadpole in metamorphosis has been amply described (Taylor and Kollros, 1946; Lynn and Wachowski, 1951; Etkin, 1955; Kollros, 1961). There have been several recent examinations of the detailed structural changes in the body skin during metamorphosis (Kemp, 1959, 1961). Thus far, however, there has been little quantitative chemical information available on structural element modifications. We have examined the alterations in the various collagen fractions, free proline, and water and cellular content of both tail and back skin in order to obtain information needed for studies on collagen turnover and collagenolytic activity. *Rana catesbiana* tadpoles used in these studies were all at the same stage of development, either legless or with hind legs less than 1 cm in length.

Metamorphosis was induced by adding thyroxin in concentrations of 10^{-6} M to the aquarium water. Control animals were starved,

since it was observed that thyroxin-treated tadpoles failed to feed.

Figure 1 illustrates the marked alterations induced in *Rana catesbiana* exposed to thyroxin for 11 days. The rate at which the tail was resorbed is charted in Fig. 2.

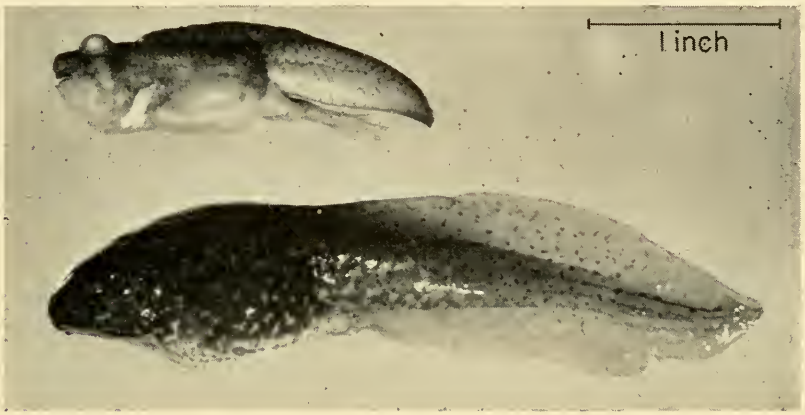

FIG. 1. Resting tadpole *Rana catesbiana;* above, tadpole after 9 days' treatment with thyroxin.

The most dramatic change in composition in the tissues of metamorphosing animals is a considerable loss of water, about 50 per cent in the fin and 25 per cent in the back skin (Table I) after 6 days' exposure to thyroxin. Collagen concentration, as well as free proline and DNA (representing cell content), was proportionately increased. Noncollagenous protein, on the other hand, showed relatively little increase in concentration. Composition changes of the whole tail fin, however, conformed closely to the visible observation of tissue resorption. Total tail fin weight diminished by three-fourths, with collagen and DNA falling approximately 50 per cent. Free proline dropped to two-thirds of the control value. The noncollagenous protein fraction* fell even lower, decreasing to one-quarter of the original level.

The loss in tissue water during metamorphosis is well known; the literature was recently reviewed by Frieden (1961).

* Represented by the protein of the extract which does not precipitate with collagen on dialysis against water.

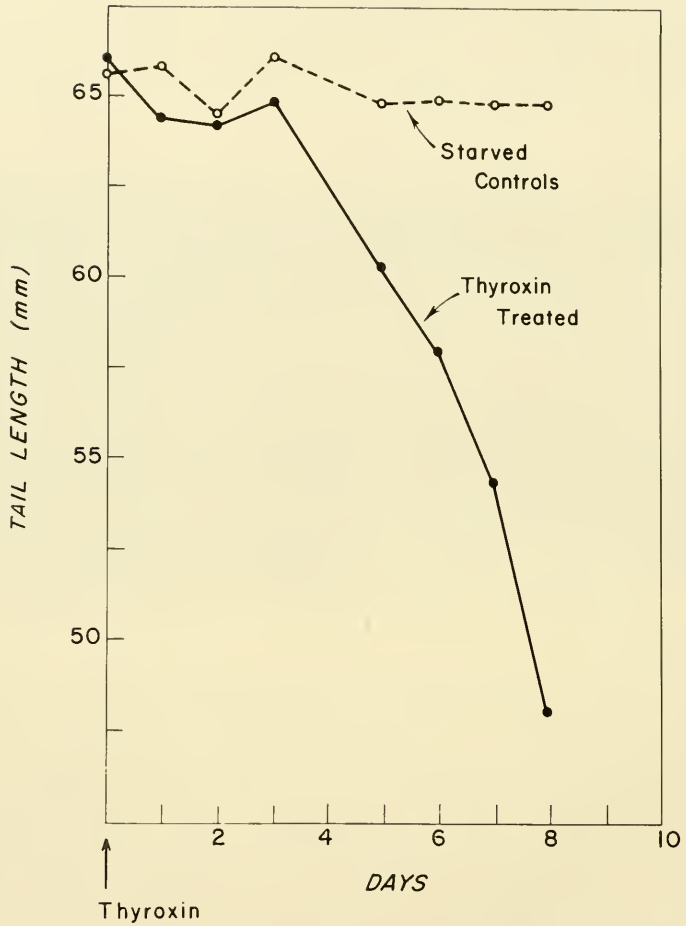

FIG. 2. Decrease in tail length of tadpoles treated with thyroxin as a function of time of exposure to the hormone. Controls were starved, since thyroxin-treated animals ceased feeding.

Comparing tail fin and back skin in the resting state, collagen concentration is fourfold higher in the latter, with free proline and noncollagen protein concentration about twice as high. There is some resorption of collagen from the back skin, associated with the change in shape and organization which occurs in this area during metamorphosis (Kemp, 1961).

TABLE I. Composition of Tail Fin and Back Skin in Thyroxin-Treated and Nontreated Tadpoles[a]

	Tail fin		Back skin	
	Control	Treated	Control	Treated
No. fins and back skins	40	40	40	40
Wet weight (mg) per organ	473	107	354	256
Per cent dry weight	5.7	10.0	7.7	10.5
	Mg/gm wet tissue		*Mg/gm wet tissue*	
Collagen	10.5	21.7	45.8	56.5
Free proline	0.07	0.16	0.14	0.16
Proline (noncollagen protein)[b]	0.20	0.21	0.37	0.44
DNA	1.5	2.7	0.63	0.77
	Mg per fin		*Mg per back skin*	
Collagen	4.9	2.3	16.2	14.5
Free proline	0.03	0.02	0.05	0.04
Proline (noncollagen protein)[b]	0.09	0.02	0.13	0.11
DNA	0.7	0.3	0.22	0.20

[a] Six days with 6×10^{-7} M thyroxin in the water.

[b] Fraction of extracted noncollagenous protein not precipitated by dialysis against water.

Metabolism of Collagen in Resting and Metamorphosing Tadpoles

The numerous studies conducted on turnover of the various collagen fractions in mammalian tissues have provided a working theory of collagen metabolism (Harkness *et al.*, 1954; Jackson and Bentley, 1959; Piez and Likins, 1957; Hausmann and Neuman, 1961; Prockop *et al.*, 1962; Gould and Manner, 1962; Green and Lowther, 1959; Gross, 1959). The tropocollagen molecule (Gross *et al.*, 1954; Schmitt *et al.*, 1955), synthesized within the fibroblast, is secreted into the intercellular space and organized into fibrils. That fraction which can be extracted in cold physiologic saline is considered to be most recently synthesized, probably existing in the tissue as loose molecular aggregates in fibrillar form. Probably the newly secreted collagen remains in the dispersed state for a very brief period in most situations, since little collagen is extractable at body tempera-

ture. Successive extractions with solvents of increasing ionic strength remove older collagen, which is more highly organized into fibrils. Acid-extractable collagen is thought to be derived from still older fibrils. The remaining collagen can be removed only by denaturation

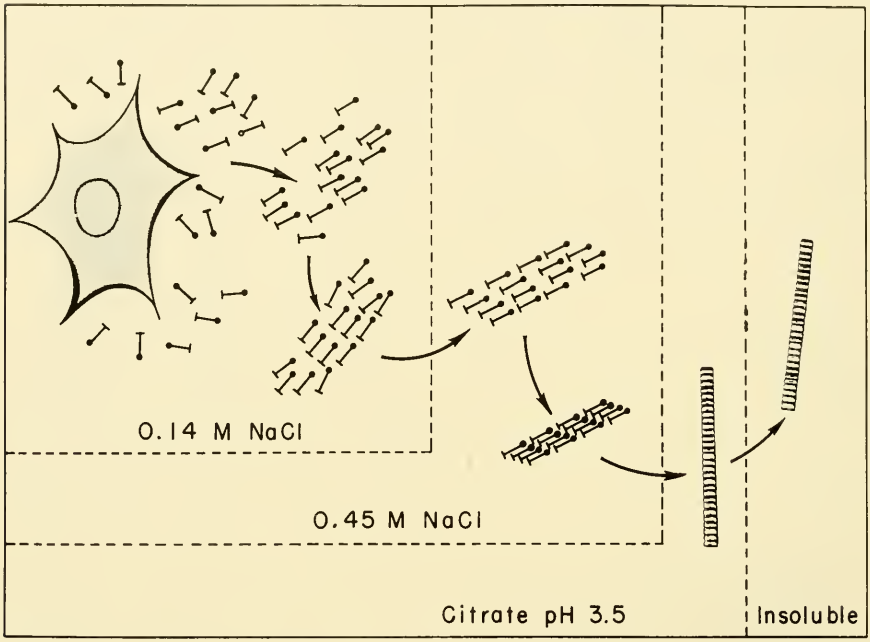

FIG. 3. Schematic representation of a hypothesis formulated to help explain occurrence of the different extractable collagen fractions. The rodlike units represent tropocollagen particles. Cold physiological saline (0.14 M NaCl) extracts the most recently formed collagen molecules (and perhaps also those resulting from physiological degradation), which are completely dissociated or in the loosest association; hypertonic salt solutions extract the same material plus older collagen in a more ordered state of aggregation; acid citrate buffer extracts all the above plus some of the older collagen in the typical fibrillar form. The insoluble fibrils were of a sufficient age so that the degree of cross linking has prevented solubilization. (From Gross, 1959.)

procedures and is most likely in a very stable, cross-linked form. This hypothesized scheme is represented diagrammatically in Fig. 3. It should be noted that these collagen fractions are operational, and not necessarily physiologically or metabolically homogeneous. For example, it has been suggested by Jackson (1957) that a portion of

the cold saline–extractable collagen might originate from degradation of nonextractable, previously deposited fibrils.

Examination of the collagen fractions reveals marked differences in distribution between back skin and tail fin, shown in Table II.

TABLE II. Collagen (Hydroxyproline) Fractions in Tissues of Thyroxin-Treated and Nontreated Tadpoles[a]

	Wet weight per organ (gm)	Av. total hydroxyproline (μmoles per fin or back skin)			
		Neutral extract (1 M NaCl)	Acid extract (0.1 M acetic acid)	Insoluble	Total
Control tail fin	0.47	0.73 (31.5%)	1.00 (42.8%)	0.59 (25.7%)	2.32 (100%)
Thyroxin tail fin	0.11	0.46 (42.8%)	0.43 (39.8%)	0.19 (17.4%)	1.08 (100%)
Control back skin	0.35	0.29 (3.5%)	2.52 (30.2%)	5.53 (66.3%)	8.34 (100%)
Thyroxin back skin	0.26	0.36 (5.4%)	3.02 (44.6%)	3.38 (50%)	6.77 (100%)

[a] Average values for 140 whole fins and 140 back skins pooled and analyzed in 7 groups.

In the former, cold neutral–extractable collagen accounts for only 3 per cent of the total, with the insoluble fibers accounting for close to 70 per cent. In the tail fin there is a roughly equal distribution between the cold neutral–extractable, the acid-extractable, and the insoluble fractions, the last accounting for only 26 per cent of the total. There is a significant reduction in the proportion of insoluble collagen in the tail fin of thyroxin-treated animals, 17 per cent as compared with 26 per cent in the controls. There is a coincident rise in neutral-extractable collagen from 32 per cent to 43 per cent. In the back skin, there is a proportionally smaller loss of insoluble collagen associated with a significant increase in the acid-extractable fraction.

A single dose of tritiated proline, 0.14 μc per gm body weight, was injected intraperitoneally into each tadpole. The thyroxin-treated animals received the label at the same time as the controls,

after 6 days of continuous exposure to the hormone. Groups of animals were sacrificed at chosen intervals up to 30 hours; the tail fins and back skins were removed, minced, and extracted three times with 5 volumes of cold 1 M NaCl, followed by three extractions with cold 0.1 M acetic acid. The extracts were dialyzed against large volumes of water, quantitatively precipitating the collagen with a small amount of globulin, leaving other noncollagenous protein in the supernatant fluid. The dialyzate contained most of the free proline and was dried by lyophilization. The material inside the bag was separated by centrifugation and the precipitates and supernatants were hydrolyzed. Hydroxyproline and proline were isolated from paper chromatograms developed with a phenol-water-ethanol solvent (Tanzer and Gross, in press). Hydroxyproline and proline are widely separated on such chromatograms and may be obtained from the paper in isotopically pure form. An aliquot part of the eluted imino acids was chemically measured and another portion counted in a liquid scintillation counter.*

It is important to note that although the labeled proline was always given in a single injection, these are not truly "pulse" experiments, since, as seen from Fig. 4, the specific activity of free proline levels off rapidly at a fairly high level, approximately 10,000 cpm/μmole even at 30 hours post injection.

Metabolism of Total Collagen

Let us first examine the incorporation of proline-H^3 into the whole collagen pool (measured as hydroxyproline) of back skin and tail fin. The data represent the sum of all three fractions.

Because of the diminishing tail size and change in water content, net incorporation of label could not be satisfactorily related to a per gram wet weight basis. As the fins and back skins were removed in a standard manner, reproducibly obtaining most of the tissue, the above measurements could best be expressed on the basis of content per "organ."

Over the 30-hour period of measurement there was only an 11 per cent loss of collagen from the tail fin and 9 per cent from the back skin. The net loss of collagen from back skin may only be ap-

* All these procedures will be described in detail in the definitive publications.

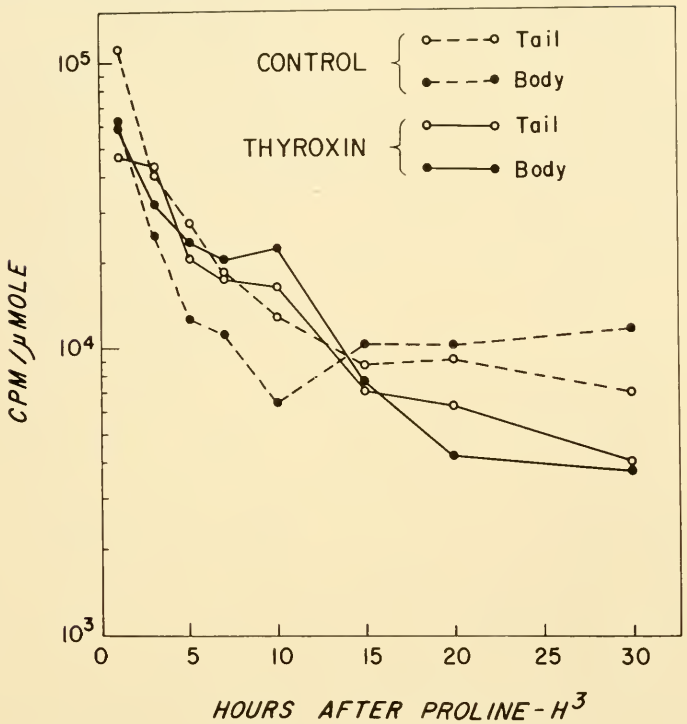

FIG. 4. Specific activity of the free proline in 1 M NaCl extracts of tail fin and back skin.

parent and probably reflects technical difficulties in making a complete dissection because of the radical changes in body and head shape.

There was greater net incorporation of labeled proline in the back skin collagen of metamorphosing animals than in the controls (Fig. 5). In the tail fin, on the other hand, the reverse occurred. The specific activity of the back skin collagen was also higher, whereas there was little difference in tail fin specific activities up to 20 hours (Fig. 6).

In the *resting* tadpole the amount of tail fin collagen was constant, and there was a progressive increase in both total and specific radioactivity over a period of 20 hours. The rates of synthesis and degradation must be the same, but whether there is some selective removal of old "cold" collagen or a nonselective random removal

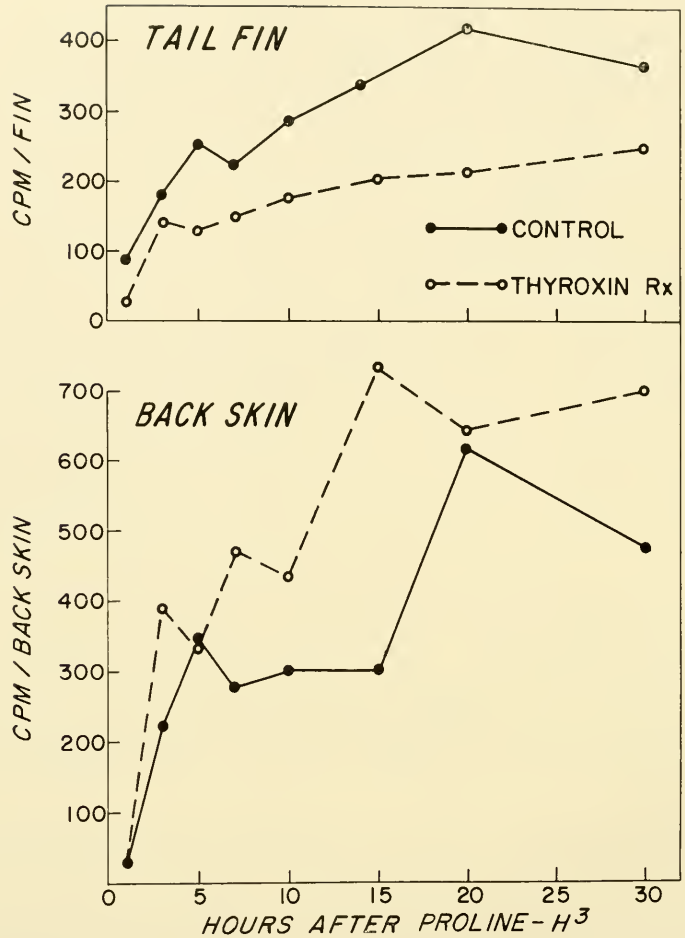

FIG. 5. Total radioactivity of hydroxyproline isolated from tail fin and back skin.

of newly deposited and old fibrils in this equilibrium state cannot be established from the data.

In the metamorphosing animal there was marked reduction in total tail fin collagen, and diminished net incorporation, but a specific activity increment identical with the normal (up to 20 hours) (Figs. 5 and 6). If the data (Fig. 5) for thyroxin-treated tail fins were "normalized" to account for the loss of about 50 per cent dry

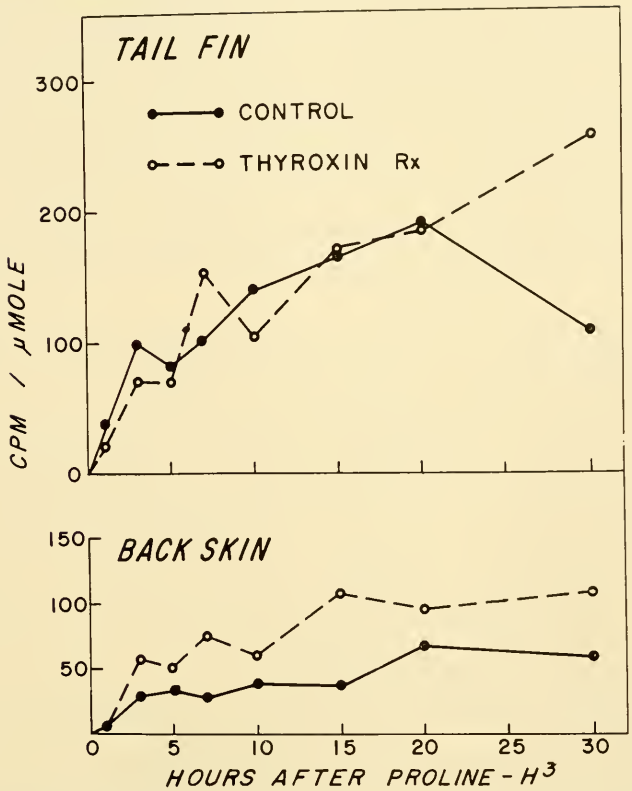

FIG. 6. Specific activity of hydroxyproline isolated from the total collagen

weight (including collagen and cells), the two curves would be superimposable. These observations suggest that the rate of synthesis of collagen was unchanged but the rate of resorption increased.

In the back skin there was an increase in both total and specific activities in the metamorphosing animals as compared with the controls (Figs. 5 and 6), indicating increased synthesis.

Metabolism of the Extractable and Insoluble Collagen Fractions

Separation of collagen into soluble and insoluble fractions gives us a more detailed picture of events. In Table II is found the amount

of collagen and percentage of the total obtained in sequential neutral and acid extracts and in the insoluble residues of control and thyroxin-treated tail fin and back skin. The amounts of collagen in the different fractions of the tail fin were roughly similar, the insoluble representing the smallest amount. In the back skin, on the other hand, there was very little neutral-extractable collagen and the insoluble fraction represented the largest portion. This latter distribution is more characteristic of that found in terrestrial mammals.

Thyroxin treatment for 6 days (in this experiment) resulted in 50 per cent diminution in the amount of neutral-extractable and insoluble collagen per tail fin and a loss of 57 per cent in the acid-extracted. By far the largest fractional loss was in the insoluble fraction. In contrast, in the back skin there was an increase in neutral- and acid-extractable collagen accompanied by a diminution in the insoluble fraction. The net result was 53 per cent loss of total collagen in the tail fin and 19 per cent loss in the back skin. Thus, partition of the different collagen fractions differed markedly between tail fin and back skin, and the influence of thyroxin on this pattern in the two tissues was also dissimilar.

In all the isotope data we were struck by the fact that incorporation of labeled proline into the neutral-extractable and insoluble collagen fractions occurred at nearly the same rapid rates.

Examination of the radioactivity of the individual collagen fractions in the tail fin discloses some interesting changes during metamorphosis. Net incorporation in the saline-extracted fraction of the control animals was about three times as great as in the insoluble residue (Fig. 7). In the resorbing tail a striking alteration is noted in the relationships of the net incorporation in the different fractions. The level of radioactivity of the neutral fraction was about equal to that of the insoluble collagen and considerably lower than in the corresponding control. The insoluble collagen net incorporation was about the same in both groups. The acid-extractable collagen was least labeled and its curve of incorporation resembled that of the insoluble fraction. Thus, the 50 per cent lower net incorporation seen in the total collagen of the metamorphosing tadpole tail fin

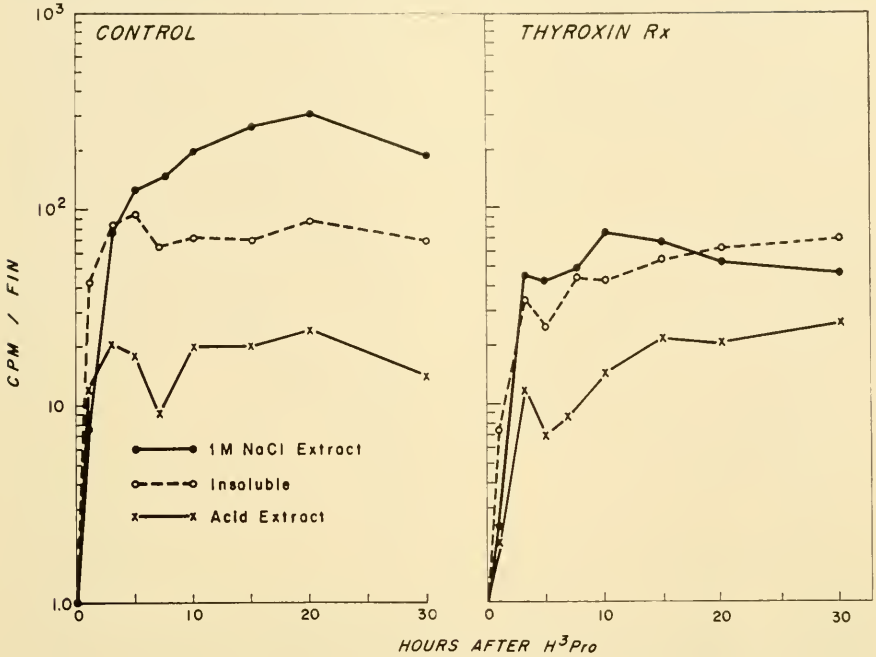

FIG. 7. Total activity of hydroxyproline isolated from the three collagen fractions of tail fin.

(Fig. 5) is due primarily to diminution in net incorporation in the saline-extracted fraction.

Looking at the specific activity relationships (Fig. 8), in the tail fin we observe that the specific activity of the insoluble component is greatly increased over that of the neutral-extractable fraction in the resorbing tail, and is also much higher than that of the control insoluble collagen. Similarly, while there is a fairly constant specific activity in the acid-extractable component in the control, there is a progressive increase in that of the other group.

It would appear likely that the rise in specific activity, coupled with a diminished amount of insoluble collagen, results from preferential removal of an unlabeled, hence older, portion of this pool, indicating that the pool is metabolically inhomogeneous. Depression of total and specific activity of the neutral-extractable fraction in

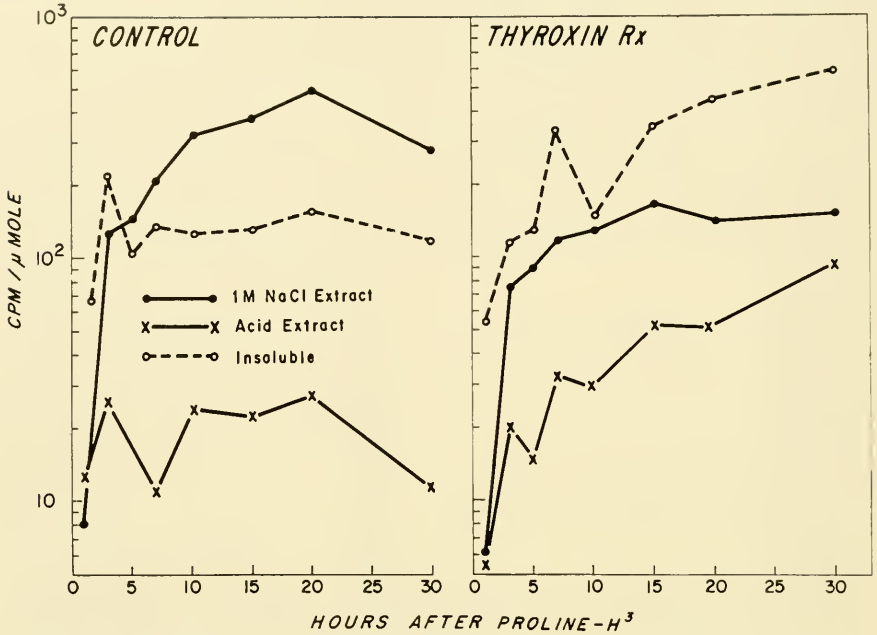

FIG. 8. Specific activity of the three collagen fractions isolated from tail fin

thyroxin-treated animals along with 43 per cent diminution of the total amount of this fraction (Table II) suggests either diminished synthesis, accelerated breakdown, or increased conversion to the insoluble pool. Diminished *net* synthesis but not *rate* would be expected because of the loss in total tissue dry weight, as discussed earlier. Since the total counts in the insoluble fraction are unchanged, there may also be an increased conversion rate.

On examination of the back skin data (Figs. 9 and 10), it appears that in the neutral-extractable fractions, total and specific activities were practically identical for control and treated groups. Specific activity of the insoluble fraction, though somewhat elevated above that of the control, did not approach the differences seen in the tail fin. The specific activity of the neutral-extracted fraction always remained considerably greater than that of the insoluble component although the total radioactivity was about the same for both. There

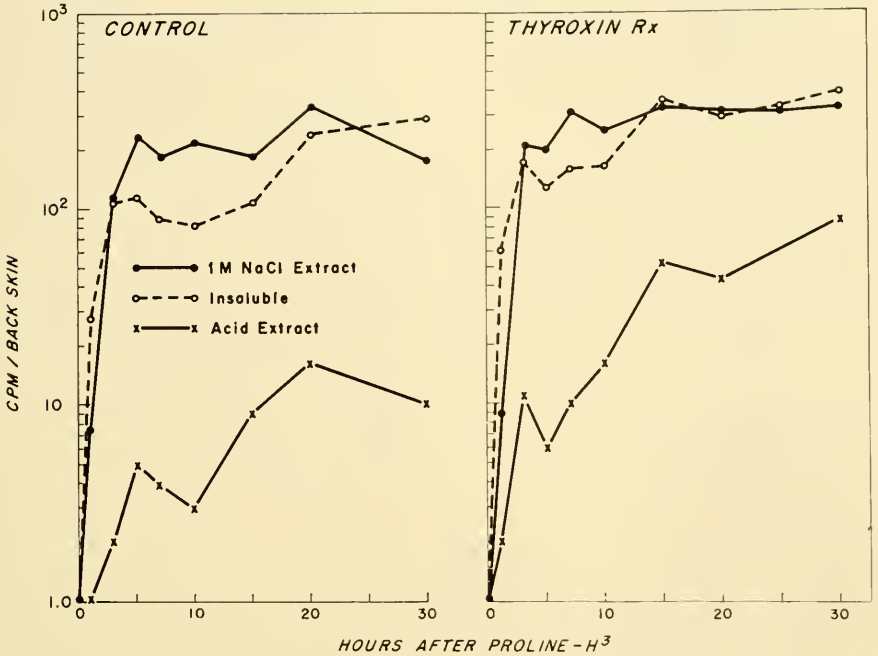

FIG. 9. Total activity of the three collagen fractions isolated from back skin

does appear to be more rapid increase in the radioactivity of the acid-extractable pool in metamorphosing tadpole back skin.

In the back skin some resorption seems to be occurring, but at a slower rate than in the tail fin. The higher rate of collagen formation in the back skin as compared with the tail fin (Fig. 5) of resting tadpoles is not altered by metamorphosis. This picture of the differences between back skin and tail fin is consistent with the observations of thickening of the dermal layer of the body during metamorphosis, associated with partial removal of the well ordered basement lamella and its replacement by a new, less well ordered terrestrial type of dermis (Kemp, 1961).

Compared with the collagen distribution and turnover data from other animal species such as rat (Harkness *et al.*, 1954) and guinea pig (Jackson, 1957; Jackson and Bentley, 1959), there are some significant differences in the tadpole. In the rodent skin the insolu-

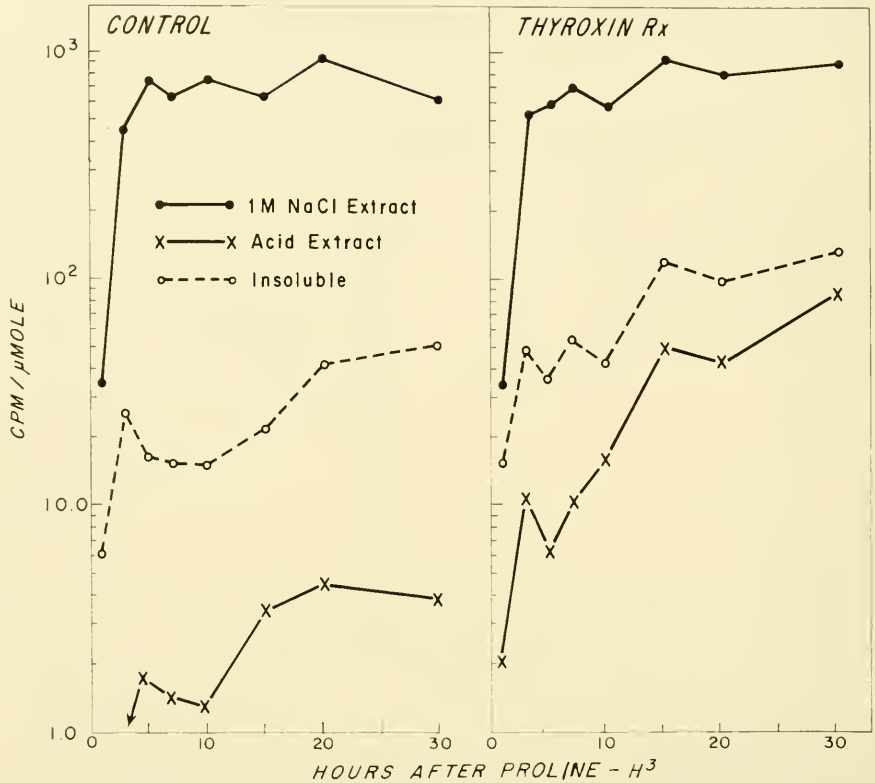

FIG. 10. Specific activity of the three collagen fractions isolated from back skin

ble fraction is by far the largest, the acid-extractable next, and the neutral-extractable the least. The situation in the back skin of the tadpole is very similar, but that in the tail fin very different, as noted. There is a striking difference in radioactivity distribution. In mammal skin the acid-extractable fraction is labeled to a greater degree and more rapidly than the insoluble, although much less than the cold neutral–extractable moiety. In contrast, in the tadpole the acid-extractable collagen of both tail and back skin is the least and slowest labeled. The shape of the curve closely parallels that of the insoluble fraction rather than that of the neutral-extractable component.

Another striking feature of the data is the nearly equal rate of

incorporation of isotope in the neutral and insoluble fractions. This suggests that either there is an extremely rapid insolubilization of the neutral-extractable collagen, or both are derived independently from a common precursor.

The fairly constant level of radioactivity obtained by all three collagen fractions over a 20- to 30-hour period is consistent with the observations of Jackson and Bentley (1959) in guinea pig skin. In their studies only the lower ionic strength cold neutral extracts showed a more rapid "turnover." The meaning is obscure, although the fairly constant high levels of labeled free proline may play a role in our experiments.

It should be kept in mind that, although induction of metamorphosis with thyroxin closely resembles the natural process, including increased thyroid activity, it is possible that some of the isotope results are a direct effect of thyroxin per se. Though this is unlikely, it has been shown, for example, that thyroid compounds in the range of concentration employed here do stimulate protein synthesis. However, at most this would only influence the results in back skin and would not affect the findings associated with tail resorption.

Collagen Degradation: Detection and Measurement of a Collagenolytic Enzyme in Tadpole Tissues

The long-recognized removal of collagen during remodeling has encouraged an intensive quest for an animal collagenase (see Mandl, 1961, for review). To qualify as a true collagenase, the enzyme should digest native collagen at physiologic pH and temperature. The usual approach has been to apply whole tissue homogenates, or fractions therefrom, to a dissolved or solid collagen substrate. Measurements of viscosity decrement or the release of hydroxyproline or free amino groups have been generally used as assay procedures, analogous to the measurement of bacterial collagenase (Gallop *et al.*, 1957). The well known proteolytic enzymes such as trypsin, chymotrypsin, the cathepsins, and papain will hardly attack native collagen under physiologic conditions (Mandl, 1961; Gross, 1958; Oken and Boucek, 1957; Hodge *et al.*, 1960; Kuhn *et al.*, 1961).

Enzymes have been isolated from mammalian white blood cells (Sherry *et al.*, 1954) and from liver lysosomal fractions (Franklin and Wynn, 1961) and uterus (Morrione and Seifter, 1962; Woessner, 1962) which attack collagen to a slight extent in the acid pH range. A type of collagenolytic activity in certain pancreatic extracts has been claimed (Houck and Patol, 1962). The only well characterized, true collagenase has been isolated from species of *Clostridium* (Mandl, 1961).

In considering the possible reasons for failure to detect an animal collagenase, we suspected that such an enzyme must be present in extremely low concentrations in highly localized regions, otherwise uncontrolled degradation incompatible with functional stability would occur. It seemed unlikely that any of the current assay procedures would be sensitive enough to detect low concentrations of this enzyme. In addition, it has been established (Gallop *et al.*, 1957) that bacterial collagenase binds to collagen fibers and is released only after digesting the protein; this being the case, the usual extraction procedures might fail to release animal collagenase from the collagen still present in the tissues. It also seemed possible that tissue collagenase might have a very short active life and either be rapidly inhibited or be degraded by other enzymes.

For these reasons we set out to detect collagenase activity by culturing small bits of tissue on a reconstituted fibrous collagen substrate. It was thought that any newly produced or activated collagenase might diffuse away from the tissue, bind to the fibrils of the substrate, digest them, and thereby reveal activity as an expanding area of lysis. This prediction was borne out and we have found collagenolytic activity in cultured tadpole tissues and in mammalian uterus and bone (Gross and Lapiere, 1962; Gross, Lapiere, and Tanzer, 1963). Figure 11 presents photographs of a typical culture of a collagen gel, showing the expanding areas of lysis around the explants of tail fin tissue.

The collagen substrates used were either acid-extracted calf skin collagen or saline-extracted radioactive guinea pig skin collagen which were purified, lyophilized, and dissolved in cold physiologic salt solutions in concentrations of approximately 0.1 per cent. The collagen polymerized to typical cross-striated fibrils (characteristic

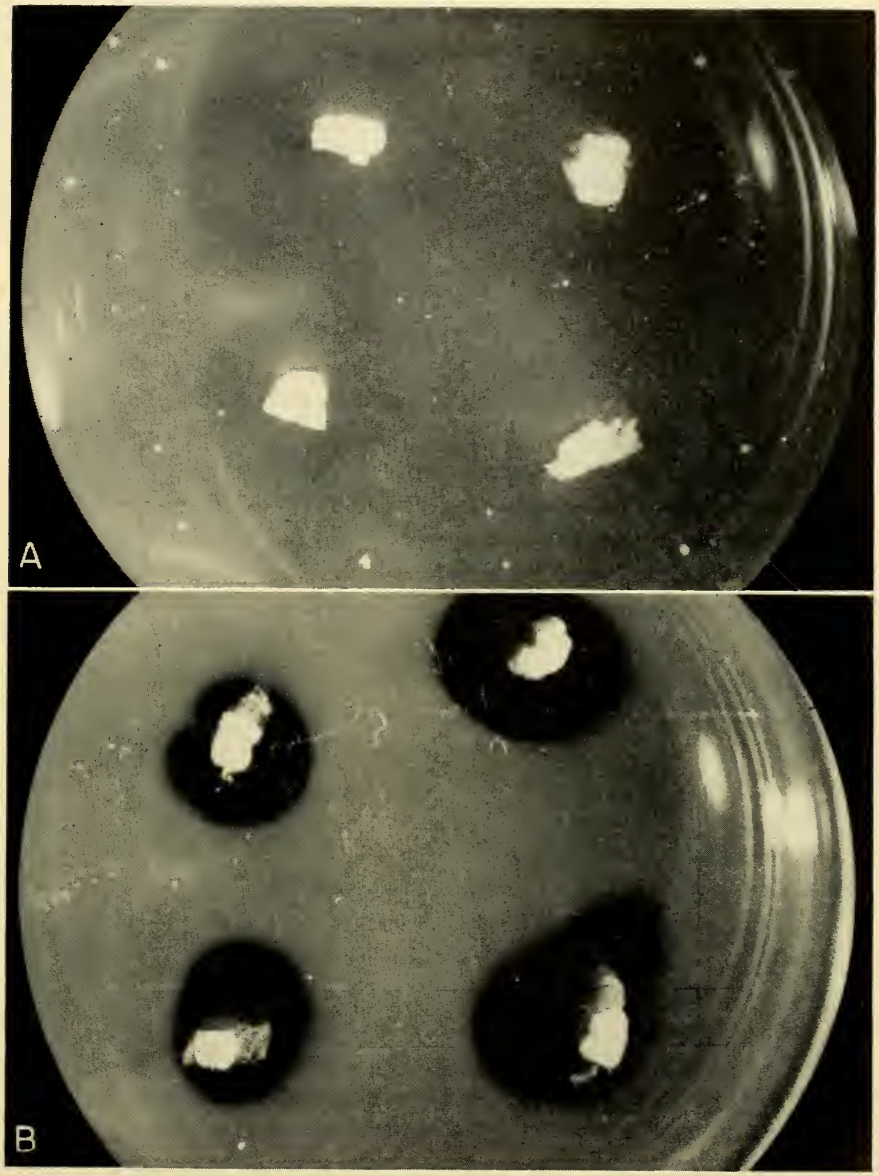

FIG. 11. Four tadpole fin explants on reconstituted calf skin collagen gel before (A) and after (B) incubation for 24 hours at 37°C.

of native structure) when warmed for short periods at 37°C (Gross and Kirk, 1958). These opalescent fibrous gels were resistant to attack by the common proteolytic enzymes (Gross and Lapiere, 1962). The tissues of tadpoles (previously sterilized by adding penicillin-streptomycin and chloramphenicol to the aquarium water) were cultured on sterile collagen gels in small ring chambers or petri dishes using a medium composed of amino acid– and vitamin-supplemented amphibian Tyrode solution. The following collagenolytic properties of tadpole tissues were recently described by the authors (Gross and Lapiere, 1962).

1. The area of lysis expanded in exponential fashion and was linearly related to the size of the tissue explant.

2. Living tissues were required for collagenolytic action. Freezing and thawing the explant prevented collagenolytic activity.

3. The pH of the medium remained in the physiologic range, and up to 80 per cent of the collagen substrate could be digested to dialyzable peptides. No free hydroxyproline was released.

4. Twelve different tadpole organs were examined for collagenolytic activity. Only four were active, tail fin and back skin, gill, and gut. These four tissues undergo the most dramatic remodeling and resorption of connective tissue during metamorphosis. The back skin showed considerably less activity than did the tail fin.

5. Tissue cultures could be used for quantitative microassay; the area of lysis was directly proportional to the release of radioactive collagen fragments when radioactive collagen substrates were used. The lytic process could be readily compared with that of a standard amount of purified bacterial collagenase placed on the gel.

Further studies have revealed changes in collagenolytic activity in the cultured tail fin tissues of animals in metamorphosis. In the first experiment, groups of tadpoles were exposed to thyroxin for 2 and 6 days. Sections of tail fin tissue approximately 1 mm^2 in area were cultured on radioactive collagen gels at 27°C. At appropriate intervals the cultures were assayed for collagenolytic activity. The results are shown in Fig. 12. Two days of treatment produced little increase in activity. After 6 days, however, when the tail had diminished by approximately 30 per cent in length, collagenolytic activity

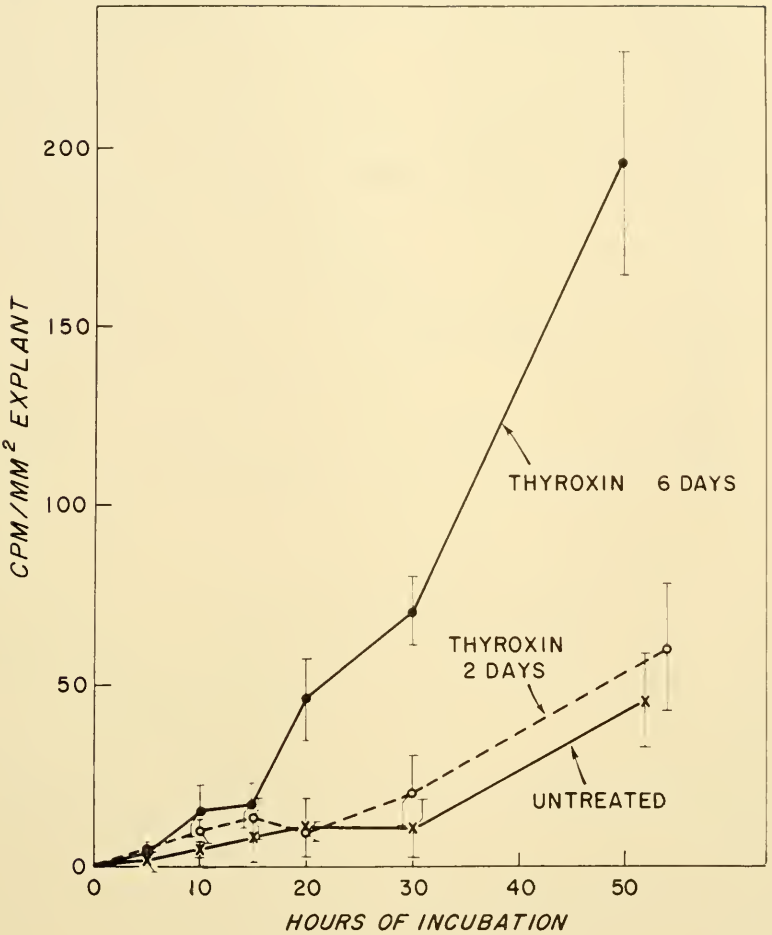

FIG. 12. Collagenolytic activity of tadpole tail fin in culture on radioactive collagen gels at 27°C.

was fourfold greater than in the control group. In several other repeat experiments the increase in activity of the treated animal tissues ranged from two to four times that of the controls.

In a second experiment, groups of tadpoles were maintained in a thyroxin-containing bath for intervals ranging from 1 to 7 days. Figure 13 illustrates collagenolytic activity in tail fin and back skin along with the decrement in tail length. The direct relationship of

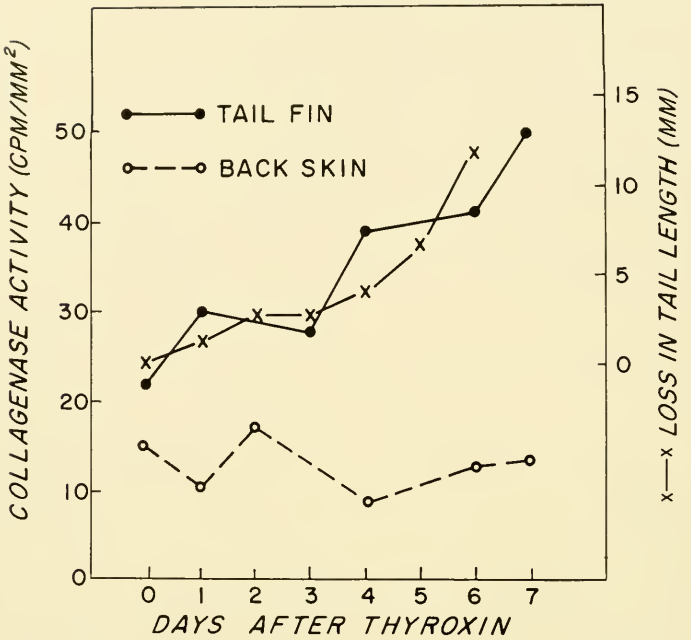

FIG. 13. Collagenolytic activity of tadpole tail fin and back skin, and decrease in tail length, as functions of time of exposure to thyroxin.

increase in enzyme activity with loss in tail length is clear; the activity of the back skin shows no relation to the change in tail size.

Isolation and purification of the tadpole collagenase from mass cultures was made possible by the development of two sensitive assay techniques which detect as little as 0.01 μg of bacterial collagenase. The first assay depends upon the release of radioactive peptides from radioactive collagen, the released fragments being measured after incubation (Lapiere *et al.*, submitted for publication). The second method is based upon decreased collagen fibril formation after incubation of a reaction mixture containing the enzyme preparation and dissolved collagen at 25°C. The temperature is then elevated to 37°C, polymerization of the collagen to an opalescent gel occurring in a few minutes. The reduction in opacity is a direct measure of collagenolytic activity (Nagai *et al.*, unpublished observations). By growing large amounts of tadpole tail fin in petri

dishes on filter paper in Tyrode solution, collagenase could be harvested from the culture medium (Nagai *et al.*, 1963). After several days of incubation, tissue fragments were removed, the breakdown products were dialyzed away, and the fluid in the bag was lyophilized to a dry powder. This material has been partially purified on Sephadex columns and compared with bacterial collagenase. Partially purified tadpole collagenase has a pH optimum between 6.5 and 7.8, is destroyed by heating between 50 and 60°C for 10 minutes, and is reversibly inhibited by EDTA. It probably has a lower molecular weight than the *Clostridium* collagenase as indicated by elution time from Sephadex columns.

Using the radioactive collagen microassay, we have reexplored the possibility of extracting substantial amounts of collagenase from tadpole tail tissues, by making extracts of control and resorbing tail fin with and without EDTA. (It has been found that the firm enzyme-substrate complex between bacterial collagenase and solid collagen can be broken with this chelating agent (Gallop *et al.*, 1957). The activity of the enzyme can be restored with calcium.) We also carefully reexamined the effect of known proteases on our reconstituted collagen substrate (Table III). We have found a small but definite amount of digestion of reconstituted collagen fibrils by very high concentrations of trypsin, chymotrypsin, papain, elastase, and pronase. This activity had to be differentiated from that of collagenase in order to evaluate the true effect of tissue extracts. It was noted that, unlike that of collagenase, the action of the other proteolytic enzymes was not progressive with time but was complete within 1 to 2 hours. Moreover, considerably larger amounts of enzyme were required for detection of this minimal activity, except for pronase, which was effective at lower concentrations. A second significant difference between true collagenolytic action and that of the other enzymes was found in response to EDTA. Both bacterial and tadpole collagenase showed complete reversible inhibition by low concentrations of EDTA, whereas the other proteases were only slightly affected (Table III). By tabulating the fractional inhibition caused by EDTA (last column, Table III) we have a sensitive index whereby collagenase activity may be differentiated from nonspecific

TABLE III. Collagenolytic Activity of Proteases and Tissue Extracts

		Cpm released from collagen substrate after 3 hours at 37°C[a]			
		EDTA C	No EDTA C_0	Δ	$\frac{C_0 - C}{C}$
Bacterial collagenase[b]	0.1 μg	1	252	251	251
Tadpole collagenase[b]	0.2 mg	6	497	491	82
Trypsin	1 mg	56	96	40	0.7
Chymotrypsin	1 mg	164	171	7	0.04
Pronase	10 μg	153	198	45	0.3
Cathepsin C + SH	3 units	6	4	0	0
Papain + SH	1 mg	160	170	10	0.06
Elastase	0.5 mg	100	117	17	0.2
		Cpm released after 24 hours			
Tadpole fin extract[c]					
1 day thyroxin Rx		68	113[d]	45	0.7
2 days " "		66	98	32	0.5
3 " " "		57	83	16	0.3
4 " " "		59	82	23	0.4
5 " " "		74	102	28	0.4
6 " " "		47	93	46	1.0
Tadpole body skin					
1 day thyroxin Rx		70	90	20	0.3
2 days " "		61	92	31	0.5
4 " " "		50	67	17	0.3
5 " " "		83	102	19	0.2
6 " " "		68	81	13	0.2

[a] Collagen substrate in each tube contains 810 cpm. Total volume is 0.6 ml.

[b] Purified *Cl. histolyticum* collagenase (courtesy of Dr. P. Gallop) and crude tadpole collagenase (lyophilized tail fin culture medium).

[c] 100 μl of extract representing 20 mg of fresh tissue homogenate in 0.12 M NaCl + 0.008 M EDTA.

[d] Excess of Ca^{++} added prior to assay.

protease activity. Values greater than 1 which increase with time of incubation are indicative of the presence of collagenase activity. Extracts of fresh tail fin and back skin from nonmetamorphosing and thyroxin-treated animals have, thus far, revealed only proteolytic type activity on collagen.

Discussion

The accretion or diminution of structural elements such as collagen is probably accomplished by altering the balance between rates of synthesis and rates of degradation. The manner in which this might occur is not yet known. The present studies suggest that where rapid collagen removal occurs, as in the resorbing tadpole tail, there is an increase in degradative activity. Synthetic activity seems to remain constant.

It is of interest that there is substantial collagenolytic activity in cultures of nonmetamorphosing tadpole tissues but such activity is essentially nondetectable in fresh tissue extracts. It may be that the culture technique allows the tissue to reveal its full potential, which it cannot do under *in vivo* restraints. Such hypothetical inhibitors were not found when large amounts of tadpole tissue extracts were added to the culture medium to prevent the appearance of collagenase. Earlier experiments (Gross and Lapiere, 1962) indicate that there is no storage of the active enzyme in the tissues, but that its accumulation in culture is a result of new synthesis or activation of a zymogen molecule. The possibility of induction or inhibition by substrate or its breakdown products requires further inquiry.

The increase in collagenolytic activity in cultures of metamorphosing tail fin ranged from two- to fourfold in repeated experiments. This increment might be largely ascribed to an increased concentration of enzyme-producing cells rather than to increased synthesis per cell. The concentration effect might result from the loss in tissue water. We propose that collagen resorption increases in metamorphosis because the cells making collagenase carry the enzyme to the substrate when they invade the collagenous basement lamella.

We hypothesize from these metabolic studies that the old insoluble collagen framework is preferentially removed and replaced continuously by a new insoluble scaffolding. It is possible that either specific cells responsible for removal can select out the older fibrils or the newly deposited fibrils are protected in some manner from attack by these cells. The ground substance, which presumably is produced

in concert with new collagen, may provide a protective coat. There is some evidence to indicate that newly formed collagen (reticulin) is surrounded by ground substance not found in older mature collagen bundles (Robb-Smith, 1957). At this stage of the game there are other equally plausible hypotheses. Hay and Revel (1963), using radioautography, have noted that newly deposited collagen first becomes evident in the regenerating basement lamella adjacent to the epithelial cells. During thyroid-induced tail resorption we (Usuku and Gross, unpublished observations) noted the early invasion of the deeper layers of the lamella by mesenchymal cells with local collagen disruption; only later were the more peripheral, presumably newly deposited, collagen layers attacked. There is also the possibility that the spreading apart of the deeper collagen layers early in resorption is a result of a shift of water into this layer. Loosening of the structure might then facilitate the entrance of the mesenchymal cells.

The differences in collagen turnover between back skin and tail fin suggest that the same stimulus, thyroxin, affects skin in two adjacent regions in different ways. There was no detectable increase in collagenolytic activity in the back skin, nor was there nearly the same degree of dehydration. The net incorporation of label in the back skin of thyroxin-treated animals was greater than that in the normal, in sharp contrast to the picture in the tail tissues. We know from older studies such as those of Clausen (1930) that body skin transplanted to the tail does not resorb during metamorphosis, whereas tail fin transplanted to the back undergoes resorption at the same rate as in its parent site. There may be some difference in the properties or composition of the collagen of the tail fin and the back skin to account for this observation. Certainly there is a marked contrast in collagen concentration and distribution between these two sites. Thus far, examination of denaturation temperature indicates that the stability of the acid-extracted collagen in the two regions is identical, and even young and old bullfrog collagens are no different in this respect. Perhaps other analyses may demonstrate a difference which could alter susceptibility to collagenase. However, it seems more likely that the cells responsible for the reorganization of the tissues in different regions respond

differently to thyroid hormone. Perhaps in the loci where very intricate remodeling takes place, as in the mouth parts, the differential response to thyroid hormone in highly localized regions plays a key role. By analyzing small *in vitro* systems we hope to be able to map out regions of differential response and ultimately determine the nature of this selectivity.

It might also be asked whether the cells responsible for collagen synthesis can produce collagenase or whether there is segregation of function in different cell types. In either case one wonders what the stimuli are which tell the cells to synthesize or digest collagen. Would both potentialities exist in the same cell or would there be further specialization? At the present time we have no idea as to which cells produce collagenase. We hope to answer the question with cell suspensions and single-cell cultures.

With regard to the behavior of mesenchymal tissues in morphogenesis, it is conceivable that the extracellular framework plays a role in limiting growth and form of an organ, either as competitor for space, or as a contact inhibitor. If this turns out to be the case, then the remodeling of mesenchyme under hormonal control may have significance beyond that of a model system.

Summary

Resorption and new formation of large collagen-containing structures have been explored at the chemical level in a controllable remodeling system, the metamorphosing and resting tadpole. Experiments on collagenolytic activity and metabolic turnover of collagen have been performed in thyroxin-induced metamorphosis, comparing tadpole tail fin collagen (which is completely removed during metamorphosis) with back skin collagen (which undergoes increased production) within the same animals.

The most dramatic change in composition in the tissues of metamorphosing animals is a considerable loss of water, with collagen and cell concentrations proportionately increased.

Tritiated proline was injected intraperitoneally into thyroxin-treated and control animals. There was greater net incorporation of labeled proline in the back skin collagen of metamorphosing animals

than in the controls, whereas in the tail fin the reverse occurred.

In the back skin there was an increase in both total and specific activities in the metamorphosing animals as compared with the controls, suggesting increased synthesis. The elevated specific activity in the face of diminished total collagen suggests a preferential attack on the older "cold" collagen.

Partition of the different collagen fractions differed markedly between tail fin and back skin, and the influence of thyroxin on this pattern in the two tissues was also dissimilar.

In the resorbing tail a striking alteration was noted in the relationships of the net incorporation in the different fractions. The specific radioactivity of the neutral-extractable fraction was well below that of the insoluble collagen and was also considerably lower than in the corresponding control. A rise in specific activity, coupled with a diminished amount of insoluble collagen, appeared to result from preferential removal of an unlabeled, hence older, portion of a metabolically inhomogeneous pool.

We speculate from these metabolic studies that the old insoluble collagen framework is preferentially removed and replaced continuously by a new insoluble scaffolding. The newly deposited fibrils may be protected in some manner from attack by the enzyme. Perhaps the ground substance, produced in concert with new collagen, may provide a protective coat. Alternatively, the older fibers are attacked first because of geographic proximity to the cells with lytic power.

Collagenase activity was tested by culturing small bits of tissue on a reconstituted fibrous collagen substrate. Living tissues were required for collagenolytic activity. Of twelve different tadpole organs examined for collagenolytic activity, four were active, namely tail fin and back skin, gill, and gut, i.e., organs which undergo the most dramatic remodeling and resorption of connective tissue during metamorphosis.

Where rapid collagen removal occurred, as in the resorbing tadpole tail, there was an increase in degradative activity. This effect might simply be due to a concentration of enzyme-producing cells as a result of tissue water loss. The increased lysis of collagen may be ascribed to the invasion of the collagenous basement lamella by

the lytic cells, i.e., they carry the enzyme to its substrate. It seemed unlikely that storage of the active enzyme in the tissue occurred. Its accumulation in culture appeared to be a result of new synthesis or activation of a zymogen molecule.

By growing large amounts of tadpole tail fin in petri dishes on filter paper in Tyrode solution, collagenase could be harvested from the culture medium. Partially purified tadpole collagenase had a pH optimum between 6.5 and 7.8, was destroyed by heating between 50 and 60°C for 10 minutes, and was reversibly inhibited by EDTA.

A small but definite amount of reconstituted collagen fibrils was digested by very high concentrations of trypsin, chymotrypsin, papain, elastase, and pronase. Unlike that of collagenase, the action of other proteolytic enzymes was not progressive with time but was complete within 1 to 2 hours; and considerably larger amounts of enzyme were required for detection of this minimal activity. Both bacterial and tadpole collagenase showed complete reversible inhibition by low concentrations of EDTA, whereas the other proteases were only slightly affected.

It seems likely that the cells responsible for the reorganization of the tissues in different regions respond differently to thyroid hormone. Perhaps in the loci where very intricate remodeling takes place, as in the mouth parts, the differential response to thyroid hormone in highly localized regions plays a key role.

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